The effects of salinization on freshwater fishes of the Northern Great Plains

A Thesis Submitted to the College of Graduate and Postdoctoral Studies in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Department of Biology

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By

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Abstract

Salinization poses a threat to many inland aquatic ecosystems. Therefore, it is important to understand the potential implications of salinization on aquatic systems. Saline water bodies are prevalent in the Northern Great Plains of North America—an area that includes portions of Alberta, Saskatchewan, and several northern states in the United States. In this area, saline water bodies provide an environmentally important habitat for a variety of species. Salinization, both natural and anthropogenic, is predicted to increase in the Northern Great Plains in the future, but the potential effects on aquatic organisms remain largely unknown. To investigate these potential impacts, I first established lethal levels of salinity exposure in fathead minnows (*Pimephales promelas*), northern pike (*Esox lucius*), and walleye (*Sander vitreus*). This experiment also gave me a framework for determining relevant concentrations with which to test the effects of sub-lethal salinity exposure on fathead minnows. After deciding appropriate levels, I exposed minnows to one of three sub-lethal salinity concentrations crossed by one of three alarm cue concentrations—an indicator of imminent predation risk. I found that salinity reduced the overall intensity of antipredator behaviour in the highest salinity concentration, and also eliminated threat-sensitive responses at the intermediate salinity concentration. I then tested the effects of salinity on reproductive endpoints, both egg-based and behavioural, in fathead minnows. I found impacts on egg-based endpoints at all salinity levels, even the relatively low 1000 parts per million (ppm) concentration. I also found significant reproductive behaviour modification in the highest salinity treatment. I was then interested in determining an adequate acclimation point at which reproductive endpoints of minnows held in saline water matched those of minnows held in dechlorinated tap water. My data suggest that the acclimation point is approximately 14 weeks in 1000 ppm salinity, though it should be noted that my scope of inference may be limited by small sample sizes. Finally, I investigated second generation effects of minnows spawned, hatched, and reared in low-level salinity. I found no effect of salinity on morphology or antipredator responses.

Acknowledgments

I dedicate this thesis to my wife, Jasmine. Even though you often thought it would never end, thank you for supporting (or at least tolerating) my grad student lifestyle for the past several years. I certainly couldn't have done this without you. Thanks for getting access to elusive research papers, keeping food on the table, and providing a nice place to live (even in Saskatoon's crazy housing market). Additionally, thanks for providing me with a route to Canadian citizenship!

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Format of the Thesis

This thesis has been organized as a manuscript-style thesis. As a result, there is repetition of information among the chapters.

Chapter 2 is a report prepared by Mike Pollock for the Saskatchewan Watershed Authority (now the Water Security Agency) to address concerns over the impact of saline water spilling from Lake Houghton to Lake Lenore. Components of the original manuscript which are not related to fish have been removed. For this study, I undertook the experiments, collected and organized data, and analyzed part of the data.

Pollock, M. S., Hoover, Z., Ferrari, M. C., Chivers, D. P., & McMaster, G. (2010). Tolerance of juvenile northern pike (Esox lucius), juvenile walleye (Sander viteus), adult fathead minnows (Pimephales promelas) and zooplankton to representative environment levels of total dissolved solids: Saskatchewan Watershed Authority.

Chapter 3 was published in Chemosphere.

Hoover, Z., Ferrari, M. C. O., & Chivers, D. P. (2013). The effects of sub-lethal salinity concentrations on the anti-predator responses of fathead minnows. *Chemosphere, 90*(3), 1047-1052.

Chapter 4 was published in Science of the Total Environment.

Hoover, Z., Weisgerber, J. N., Pollock, M. S., Chivers, D. P., & Ferrari, M. C. O. (2013). Sub-lethal increases in salinity affect reproduction in fathead minnows. *Science of the Total Environment, 463–464*, 334-339.

Chapter 1: Introduction

1.1 Inland Saline Water Bodies

Inland saline water bodies, which are common in many parts of the world, represent an important habitat for aquatic animals and migratory birds. While saline water bodies are somewhat undervalued resources, they are often important commercial and recreational sites (Bowman & Sachs, 2008; Williams, 2001). Inland saline water bodies typically occur in endorheic drainage basins (closed drainage basins with no outflow) in areas of low precipitation and high evaporation (Hammer, 1978b). The Northern Great Plains of North America, an area encompassing portions of Alberta, Saskatchewan, and several northern states in the United States, contain thousands of saline water bodies (Last & Slezak, 1988). One of the first scientific surveys of the region occurred in 1875, and reported the readily apparent physical characteristics of saline lakes and ephemeral water bodies (reported in Last, 1992). The first truly comprehensive and systematic survey of saline water bodies in Saskatchewan was undertaken by Rawson and Moore (1944), and occurred between 1938 and 1942. In their survey, Rawson and Moore discussed the physical, chemical, and biological characteristics of approximately 60 lakes in Saskatchewan which they considered saline. Since that time, the accepted definition of a saline lake has changed from a salinity concentration of 300 parts per million (ppm) to a concentration of 3000 ppm (Williams, 1964). Thirty years later, another study updated Rawson and Moore's research, summarizing the characteristics of approximately 60 permanent and ephemeral saline lakes in Saskatchewan (Hammer, 1978a, 1978b). Both the 1944 and 1978 studies found the saline lakes of Saskatchewan to be dominated by sulfate, magnesium, and sodium, and Hammer found a salinity range of ≈ 3,000 - 342,000 ppm (Hammer, 1978b; Rawson & Moore, 1944).

Lake Lenore is an example of a typical Saskatchewan saline lake. It has a published historical total dissolved solids (TDS) range of 6360 ppm (Hammer, 1978b) to 5200 ppm (Hammer, Sheard, & Kranabetter, 1990), and is currently ≈ 2700 ppm (fall 2016, Water Security Agency, personal communication). Lake Lenore shares a basin with many lakes, some of which have much higher salinity. In the recent past, this basin received above average precipitation. This extra precipitation resulted in higher water levels, causing nearby Lake Houghton (approximately 14000 ppm TDS) to overflow into Lake Lenore. In 2008, Fisheries and Oceans Canada stopped the natural flow of water between the lakes,

due to fears of potential negative impacts on the aquatic ecosystem. However, it was unknown at that point whether increasing salinity would negatively affect organisms in Lake Lenore.

1.2 Salinity and the Environment

Salinization of inland water bodies can occur in two ways. Natural, or primary salinization, has no anthropogenic basis. It is typically caused by the accumulation and concentration of salts via ice-cover, weathering of rocks and soil, and the process of evaporation and subsequent concentration of salts. Salts accumulate in endorheic basins because there is no outlet to the ocean. Therefore, salts deposited in these areas are not washed out, and instead accumulate. For an overview of the paleolimnological characteristics which resulted in the deposition of salts in several basins in the Northern Great Plains, see Last (1988).

Anthropogenic, or secondary salinization, is caused by human activities, and may be more rapid than primary salinization (Cañedo-Argüelles et al., 2013; Trombulak & Frissell, 2000). Secondary salinization can have many causes, including clearing of natural vegetation for development, discharge of wastewater, irrigation, runoff from streets and agriculture, erection of dams, and mining activities (Carpenter, Stanley, & Vander Zanden, 2011; Williams, 2001). Confounding both primary and secondary salinization, global climate change almost certainly plays an exacerbating role, but the link is notoriously difficult to discern (Covich et al., 1997; Pratchett et al., 2011).

1.3 Salinity and Aquatic Ecosystems

Salinization, specifically secondary salinization, has been called the greatest cause of environmental degradation in some aquatic ecosystems (James, Cant, & Ryan, 2003). This may seem an extreme statement, but increasing salinity has been shown to cause shifts in biotic communities, limit biodiversity, exclude less tolerant species, and cause acute or chronic effects at specific life stages (Kaushal et al., 2005; Weber-Scannell & Duffy, 2007). In fact, even if increases in salinity are relatively short-lived and normal conditions return, vulnerable populations may not be able to recover (Wedderburn, Barnes, & Hillyard, 2014). Driver and Peden (1977) report that salinity concentrations of permanent saline water bodies in the Canadian Prairies can vary seasonally up to 20%. Therefore, most established populations, particularly those which have successfully experienced such salinity

fluctuations in the past, should be able to accommodate mild natural salinization. However, when this natural variation is combined with unpredictable rates of secondary salinization, salinity concentrations may become intolerable, compromising the ability of organisms to cope.

Increases in salinity have been shown to negatively impact invertebrates (Cormier, Suter, & Zheng, 2013; Piscart et al., 2006), amphibians (M. G. Brown et al., 2012; Cañedo-Argüelles et al., 2013; Chinathamby, Reina, Bailey, & Lees, 2006), and fishes. The vast majority of research into the effects of salinity on fishes involves survival studies (typically LC50) and the physiological mechanisms of osmoregulation. For a review of osmoregulation in teleost fishes, see Evans (2008). Fewer studies have examined the behavioural impacts of sub-lethal salinity exposure. The distinction between physiological tolerance and behavioural modification threshold is important because some studies have found changes in behaviour well below lethal levels. For instance, Whiterod and Walker (2006) found an LC50 for common carp (*Cyprinus carpio*) of 13000 ppm with behaviour modification at concentrations as low as 7500 ppm. Similarly, at sub-lethal concentrations, Alcaraz et al. (2008) found that mosquitofish (*Gambusia holbrooki*) competed less successfully with Mediterranean killifish (*Aphanius fasciatus*) for food, and Luz et al. (2008) found decreased locomotion and feeding behaviour in goldfish (*Carassius auratus*).

In addition to being a potential stressor itself, there is some evidence that salinity can increase the toxicity of organic pollutants (Noyes et al., 2009) and insecticides (Lavado, Maryoung, & Schlenk, 2011). In such cases, salinization of aquatic systems could be a much larger threat than salinization or the presence of toxicants alone.

Most research examining the impacts of salinization on aquatic organisms focuses exclusively on NaCl (for example, Bezirci et al., 2012; Pistole, Peles, & Taylor, 2008). While Na^+ and Cl^- are the major ions found in many salinized water bodies around the world, other regions can be dominated by different ions, such as the $MgSO_4$ and $NaSO_4$ dominated lakes of the Northern Great Plains. This is an important distinction because the ion ratio of saline water has been shown to have dramatic physiological effects. For instance, Mount et al. (1997) found the 96-h LC50 for fathead minnows (*Pimephales promelas*) ranged from <510 to 7960 ppm based on the ion ratio and salts present in the experimental water. Similarly, for rainbow trout (*Oncorhynchus mykiss*) and larval chironomids (*Chironomus tentans*), Chapman et al. (2000) found that the toxicity of mining effluents was not predictable based on total dissolved solids (TDS) concentration alone, but instead depended upon the combination and concentration of ions in the water.

1.4 Choice of Species

Fathead minnows were chosen as the species of study for Studies 1-5. Fathead minnows are small-bodied prey fish common throughout much of central North America. Because they are hardy and tolerant of a wide range of water quality characteristics (Ankley & Villeneuve, 2006), minnows can be found in many inland aquatic systems, where they serve as important consumers and prey. In fact, the presence or absence of fathead minnows has been directly associated with changes in several biotic and abiotic factors in small lakes of the North American prairies (Zimmer, Hanson, & Butler, 2002). Due to their widespread distribution, fathead minnows are often subjected to both primary and secondary salinization—especially in the Great Plains, where saline lakes are fairly common. This lends direct ecological relevance to the studies incorporated in this thesis.

In addition to their hardiness, widespread distribution, and ecological relevance, fathead minnows were chosen for several characteristics which make them particularly well-suited for laboratory study. First, their small adult body size, approximately 4-6 cm length (Landis & Yu, 2004; Ross, 2001), allows large numbers of minnows to be housed in relatively small spaces. Second, fathead minnows have rapid development from egg to adult under optimal conditions—4-5 months (OECD, 2009; USEPA, 2002). Third, minnows have well-characterized antipredator behaviours which can be readily tested in the laboratory environment (Chivers & Smith, 1998; Ferrari, Wisenden, & Chivers, 2010). Fourth, fathead minnows have become ubiquitous in regulatory testing and research (Ankley & Villeneuve, 2006; USEPA, 2002), leading to the development of standardized laboratory protocols for testing.

In addition to fathead minnows, juvenile northern pike (*Esox lucius*) and juvenile walleye (*Sander vitreus*) were chosen for Study 1. These species were selected because they were deemed species of concern for Lake Lenore by the government agencies involved (A.2 Fish test species). Juveniles were chosen not only because adults are large-bodied, and therefore difficult to maintain in the laboratory setting, but also because smaller fishes may be less capable of successfully osmoregulating in saline environments than larger fishes (Mojazi Amiri et al., 2009).

1.5 Fathead Minnow Antipredator Behaviour

Predator avoidance is an essential component of survival for any organism. Therefore, it should be unsurprising that a wide variety of aquatic phyla, including Cnidaria, Platyhelminthes, Mollusca, Arthropoda, Echinodermata, Annelida, and Chordata have developed means to detect predators through chemical messengers in the environment (Chivers & Smith, 1998; Ferrari et al., 2010). Response to these chemical messengers may be either innate or learned, and the messengers themselves may include predator odour, disturbance cues (*e.g.* urinary ammonia), or alarm cues released when conspecifics are injured by predators (Ferrari et al., 2010).

Fathead minnows have well-characterized antipredator behaviours and show a remarkable ability to distinguish among a variety of chemical cues indicating risk. For example, minnows can determine the size (Kusch, Mirza, & Chivers, 2004), density, and proximity (Ferrari, Messier, & Chivers, 2006) of predators based on the odour signature of their predators. Minnows also respond to chemical alarm cues released by nearby conspecifics which have been attacked by a predator (Chivers & Smith, 1998). These cues serve as an early warning, alerting prey fish to the presence of a nearby predator. Interestingly, alarm cues are produced by cells which seem to have no direct antipredator function. Instead, these cells serve an immune function in the epidermis, and alarm cues released by these cells during attack serve the secondary function of alerting fish to the presence of a predator (Chivers et al., 2007). Typical antipredator behaviours in minnows include a decrease in movement and an increase in shoaling and vigilance (Chivers & Smith, 1998). Fish adequately displaying these behaviours in the presence of a predator may show increased survival (Mirza & Chivers, 2001).

Fathead minnows have been shown to exhibit threat-sensitive responses to chemical alarm cues and predator odours (Ferrari et al., 2005; Kusch et al., 2004). This means minnows increase the intensity of their antipredator response when they perceive an increased threat. Essentially, fathead minnows modulate their antipredator response to match the perceived level of threat, in accordance with Helfman's threat-sensitive predator avoidance hypothesis (1989). This confers a fitness advantage: time and resources are not wasted due to excessive or insufficient antipredator behaviours (Lima & Dill, 1990).

1.6 Salinity and Reproduction in Fishes

In general, there has been little research examining the effects of increasing salinity on reproduction in stenohaline (aquatic organisms that can only tolerate relatively modest changes in salinity) freshwater fishes. One such study found reduced sperm motility in common carp (*Cyprinus carpio*) at approximately 8000 ppm (Whiterod & Walker, 2006). Most of the studies concerned with freshwater fishes examine fertilization success, egg hardening, and the early life stages of anadromous salmonids (for example, Brix et al., 2010; Chapman et al., 2000). One of these studies found decreased fertilization success and decreased viability at the alevin stage in salmonids, with fertilization effects beginning as low as 250 ppm (Stekoll et al., 2009).

Fathead minnows can be induced to readily reproduce in the lab, and can spawn continuously for several months if conditions are maintained (Ankley & Villeneuve, 2006). Minnow pairs may routinely produce up to 500 eggs per female over a 21-d period (USEPA, 2002). Given their environmental relevance, ease of care, and ready reproduction in laboratory settings, short-term reproductive assays have been developed for minnows by both the United States Environmental Protection Agency (USEPA) and the Organisation for Economic Cooperation and Development (OECD). The short-term reproductive assay is used as an environmental impact assessment tool for chemicals which have been introduced into the aquatic environment (OECD, 2009; USEPA, 2002). This assay is generally used to test the effects of endocrine-active substances which alter the sexual characteristics of the test fish. Though these tests could be easily adapted to study salinity, few researchers have done so.

1.7 Objectives

The main objective of my research is to study the effects of salinity, specifically saline water similar to that found in many water bodies in the Northern Great Plains of North America, on stenohaline freshwater fishes, with an emphasis on the effects of sub-lethal salinity on fathead minnows. In order to achieve this objective, the five data chapters found in this thesis explore the following questions:

How does salinity affect the survival and behaviour of juvenile northern pike, juvenile walleye, and adult fathead minnows? Salinity has been shown to be lethal in many freshwater fishes, though behaviour has not been thoroughly investigated. In Chapter 2, we conducted LC50 tests with three fish

species using experimental water which mimicked the ion ratio of Lake Lenore. In addition to the standard LC50 protocol, we also included a behavioural component in order to investigate the possibility that behaviour would be impacted at sub-lethal levels.

Does sub-lethal salinity affect the antipredator responses of fathead minnows? If salinity affects the overall antipredator responses of minnows, does it also affect their threat-sensitive responses? The complex antipredator behaviour of fathead minnows has been well-characterized, but any impacts of salinity had not previously been investigated. In Chapter 3, we explored this question by conducting a 3x3 study, using three levels of salinity and three alarm cue treatments. Using this approach, we were able to answer both questions concurrently.

Does sub-lethal salinity affect reproduction in fathead minnows? Few studies have investigated the effects of salinity on reproduction in freshwater fishes. Of those studies, the vast majority are concerned with anadromous fishes, specifically salmonids. To address this lack of information in the literature, Chapter 4 investigated the effects of sub-lethal salinity on fathead minnow reproduction across four salinity levels using a short-term reproductive assay. Because reproductive behaviour in minnows is well-characterized and easily observable in the lab, both egg-based and behavioural endpoints were used.

Using reproductive endpoints as a measure of acclimation to increased salinity, are fathead minnows able to acclimate to 1000 ppm? Given that we were able to show an impact of salinity on minnow reproduction at the relatively low concentration of 1000 ppm in Chapter 4, Chapter 5 attempts to determine if minnows are capable of acclimating to increased salinity, using reproductive endpoints as a measure of acclimation. To this end, I held minnows in either dechlorinated tap water or 1000 ppm salinity for increasingly long periods of time and used short-term reproductive assays to compare egg-based endpoints between treatments.

Does sub-lethal salinity affect the morphology and/or antipredator behaviour of second generation fathead minnows? Chapter 6 explores generational effects on minnows spawned, hatched,

and raised in 1000 ppm salinity. Minnows were produced in dechlorinated tap water and 1000 ppm salinity during the studies included in Chapter 5. Those second generation minnows were examined for morphological differences. Then, the same minnows were exposed to one of two alarm cues in a 2x2 design.

Chapter 2: Study 1—Impact of salinity on survival of three fish species, *Esox lucius*, *Sander vitreus*, and *Pimephales promelas*

This manuscript was prepared by Mike Pollock for the Saskatchewan Watershed Authority (now the Water Security Agency). In addition to the information contained in Chapter 2 of this thesis, the original manuscript contains a study on the impacts of salinity on several zooplankton species. The zooplankton study and any references to it have been removed from the manuscript.

For the studies contained in Chapter 2, I undertook the experiments, collected and organized data, and analyzed part of the data. The published version has been altered to match the formatting of this thesis.

Pollock, M. S., Hoover, Z., Ferrari, M. C., Chivers, D. P., & McMaster, G. (2010). Tolerance of juvenile northern pike (*Esox lucius*), juvenile walleye (*Sander viteus*), adult fathead minnows (*Pimephales promelas*) and zooplankton to representative environment levels of total dissolved solids: Saskatchewan Watershed Authority.

2.1 Executive Summary

The current study was conducted between June and August of 2009 at the University of Saskatchewan's R.J.F. Center for Aquatic Ecology. The study was initiated in response to concerns over the potential impact of salinity (i.e. total dissolved solids [TDS]) in Lake Lenore due to inflow from Lake Houghton, as well as the potential for future increases due to lake evaporation. The study was conducted by Zach Hoover as part of a master's degree program and in accordance with the collaborative proposal prepared by the Saskatchewan Watershed Authority, Fisheries and Oceans Canada, Ministry of the Environment and the University of Saskatchewan. The current study had two goals: 1) to determine the concentration of TDS that would cause 50% mortality (*i.e.* LC50) in juvenile pike (*Esox lucius*), juvenile walleye (*Sander vitreus*), and adult fathead minnow (*Pimephales promelas*); 2) to determine the concentration of TDS that will cause a significant effect on fish behaviour. Salinity concentrations used in both components were identical and included 250 ppm (control), 1000 ppm, 2000 ppm, 4000 ppm, 6000 ppm, 8000 ppm, and 16000 ppm. To ensure study relevance, the ionic composition of the TDS was formulated to mimic that of Lake Lenore. Results of the study indicate that the 96-hour LC50 of pike, walleye, and fathead minnows were 11627 ppm, 8316 ppm, and 11627 ppm, respectively. Behaviour proved to be a more sensitive measure in pike with significant decreases in movement at 2000 ppm, while walleye displayed no effect on behaviour, and minnows displayed changes in behaviour at high levels (6000 ppm and 8000 ppm). Given our results and the current TDS levels at Lake Lenore (4000 ppm), it can be concluded that the fish populations within Lake Lenore are not at risk of a significant die off due to inflows from Houghton Lake, but if TDS increases may display behavioural changes.

2.2 Introduction

This report summarizes four experiments conducted by the University of Saskatchewan (U of S) investigating the potential impacts of total dissolved solids (TDS) on aquatic communities relevant to Lake Lenore. This series of experiments was initiated and designed in response to concerns that a potential rise in TDS in Lake Lenore could negatively impact the aquatic community. As such, species used in the study are relevant to those found in Lake Lenore as is the specific ion ratio used. The current study examined the impacts of TDS on northern pike (*Esox lucius*), walleye (*Sander vitreus*), fathead minnows (*Pimephales promelas*). The outcome of this series of experiments will fill information gaps

needed to make informed management decisions with respect to the Lake Lenore fish population and hydrology. For a review of the known impacts of TDS on the aquatic community as well as a more thorough history of the Lake Lenore situation see the original proposal.

All studies were conducted between June and August of 2009 using young-of-the-year pike and walleye, and adult fathead minnows. Pike and walleye were provided by the Ministry of the Environment (MoE) (Contact: Jennifer Merkowsky). Fathead minnows were collected at a pond located on the U of S campus. The studies were conducted at the U of S, RJF Smith Center for Aquatic Ecology. Studies were conducted by Zach Hoover, with the assistance of Saskatchewan Watershed Authority (SWA) staff and supervised by Professor Doug Chivers (Animal Care Protocol 20090090). Studies were conducted in accordance with a collaborative proposal written by the SWA, Fisheries and Oceans Canada (DFO), MoE, and the U of S. Except where noted in the proposal, studies were based on guidelines set by the Organization for Economic Co-operation and Development (OECD, 1992).

The purpose of the study was twofold: 1) to determine the concentration of TDS that would cause 50% mortality (*i.e.* LC50) in juvenile pike, juvenile walleye and adult fathead minnow; 2) to determine the concentration of TDS that will cause a significant effect on fish behaviour.

2.3 Materials and Methods

2.3.1 Fish protocols

As previously mentioned, the study design is based on the OECD guidelines for testing fishes' acute responses to chemicals (OECD, 1992). The study proceeded as outlined in the proposal with no changes to protocol. For a complete review of the protocol please see the original proposal (Appendix A: Methods for Study 1). By way of quick review, the study proceeded as follows:

- Young-of-the-year pike (4-6 weeks in age) were harvested from Van Pattens pond upstream of Little Fishing Lake (TDS ≈ 800 ppm TDS); young-of-the-year walleye (4-6 weeks in age) were harvested from Codette Pond (TDS ≈ 400 ppm); Adult fathead minnows were harvested from Feedlot Pond located on the University of Saskatchewan campus (TDS ≈ 450 ppm)
- Following harvest, fish were maintained in the laboratory for one week to be sure the population was stable and healthy (water quality and chemistry identical to study control)

- During this acclimation period, fish were held at 16:8 photoperiod at 17°C and fed twice daily with brine shrimp (*Artemia* spp) and young-of-the-year fathead minnows (pike and walleye only)
- Following one week of acclimation, individuals were arbitrarily placed into 9 L tanks held at 20-21°C and 250 ppm TDS, and given 24 hours to acclimate
- Following acclimation, TDS levels were brought up to experimental levels over a 24-hour period (See Appendix B: Salinity Preparation for Study 1 for the masses of salts used)
- Fish were held at these levels for 96 hours
- Fish were checked every six hours for mortality
- Each fish was observed daily for a five-minute period, during which time spent moving was recorded
- Sample size for LC50 calculations and behaviour was 10
- Sample size for water quality calculations was four (one sample/day)
- All results were analyzed using SPSS v. 17.

2.4 Results

2.4.1 Fish Results

Analysis of fish data was divided into physical parameters, water quality, behavioural responses and LC50 calculation.

2.4.1.1 Physical Parameters

Given the fact that size has the ability to impact osmoregulatory ability (Mojazi Amiri et al., 2009), mass and length data were collected from all fish before they were arbitrarily assigned to treatment groups. Following treatment assignment, a one-way analysis of variance (ANOVA) was performed on weight and length to be sure treatment groups did not differ significantly (Table 2-1, Table 2-2, and Table 2-3). At completion of the study, mass and length data were collected from all surviving fish to determine if any significant decrease in length or mass occurred among treatments (see above-referenced tables).

Results from the pike study indicated that neither pre-study weight (ANOVA, $F_{(6,61)} = 0.963$, $p = 0.45$) nor pre-study length ($F_{(6,61)} = 0.820$, $p = 0.56$) differed among groups. Results indicated no significant change in length ($F_{(6,61)} = 0.67$, $p = 0.67$) or mass ($F_{(6,61)} = 1.28$, $p = 0.28$) following the four-day exposure.

Similar results were found in walleye, with no difference in pre-study length (ANOVA, $F_{(6,60)} = 0.25$, $p = 0.96$) or mass ($F_{(6,60)} = 0.99$, $p = 0.44$), nor were there differences in post-study length ($F_{(6,60)} = 0.22$, $p = 0.97$) or mass ($F_{(6,60)} = 2.14$, $p = 0.06$).

Patterns of pre-study mass (ANOVA, $F_{(6,62)} = 1.07$, $p = 0.39$) and length ($F_{(6,62)} = 0.83$, $p = 0.56$) were similar in minnows, as was post-study mass ($F_{(6,62)} = 0.98$, $p = 0.44$) and length ($F_{(6,62)} = 0.87$, $p = 0.52$).

Table 2-1. Mass and length data (mean + 1 SD) collected before and after the four-day pike/TDS exposure conducted in the summer of 2009 at the University of Saskatchewan.

Concentrations (ppm)							
Target total dissolved solids	**250**	**1000**	**2000**	**4000**	**6000**	**8000**	**16000**
Actual total dissolved solids*	270	990	2070	4130	6070	8000	17600
Initial mass (g) - no significant difference among treatments.							
Mean	1.7	1.6	1.1	1.5	1.2	1.0	1.7
SD	1.3	1.0	0.7	1.1	0.7	0.4	1.2
Final mass (g) - no significant differences among treatments**.**							
Mean	1.2	1.1	0.8	1.1	0.8	0.7	1.4
SD	0.9	0.8	0.5	0.7	0.6	0.3	1.0
Delta mass (g)							
Mean	-0.5	-0.5	-0.3	-0.4	-0.4	-0.3	-0.3
Initial Length (cm) - no significant differences among treatments.							
Mean	5.6	5.6	5.0	5.5	5.1	5.1	5.4
SD	1.0	0.8	0.7	0.8	0.9	0.5	1.0
Final length (cm) - no significant differences among treatments.							
Mean	5.6	5.5	5.0	5.5	5.2	5.1	5.4
SD	1.0	0.9	0.7	0.9	0.8	0.5	1.0
Delta length (cm)							
Mean	0.0	-0.1	0.0	0.0	0.1	0.0	0.0

* data analyzed by the Saskatchewan Research Council (SRC) from water collected at the study's end

Table 2-2. Mass and length data (mean + 1 SD) collected before and after the four-day walleye/TDS exposure conducted in the summer of 2009 at the University of Saskatchewan.

Concentrations (ppm)							
Target total dissolved solids	**250**	**1000**	**2000**	**4000**	**6000**	**8000**	**16000**
Actual total dissolved solids*	213	938	2030	3860	5560	7530	15400
Initial mass (g) - no significant difference among treatments.							
Mean	0.7	0.7	0.8	0.6	0.7	0.6	0.7
SD	0.1	0.1	0.1	0.1	0.2	0.1	0.1
Final mass (g) - no significant differences among treatments.							
Mean	0.4	0.4	0.5	0.4	0.5	0.5	0.6
SD	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Delta mass (g)							
Mean	-0.3	-0.3	-0.3	-0.2	-0.2	-0.1	-0.1
Initial Length (cm) - no significant differences among treatments.							
Mean	3.9	3.9	3.9	3.8	3.8	3.8	3.8
SD	0.4	0.2	0.1	0.4	0.2	0.3	0.2
Final length (cm) - no significant differences among treatments.							
Mean	3.9	3.9	3.9	3.8	3.8	3.9	3.8
SD	0.4	0.2	0.1	0.4	0.2	0.1	0.2
Delta length (cm)							
Mean	0.0	0.0	0.0	0.0	0.1	0.1	0.0

* data analyzed by the Saskatchewan Research Council (SRC) from water collected at the study's end

Table 2-3. Mass and length data (mean + 1 SD) collected before and after the four-day fathead minnow/TDS exposure conducted in the summer of 2009 at the University of Saskatchewan.

Concentrations (ppm)							
Target total dissolved solids	**250**	**1000**	**2000**	**4000**	**6000**	**8000**	**16000**
Actual total dissolved solids*	281	1030	2030	4120	5970	7980	16100
Initial mass (g) - no significant difference among treatments.							
Mean	1.7	1.6	2.5	2.1	2.4	2.1	2.7
SD	0.9	0.8	1.5	0.9	1.0	0.8	1.8
Final mass (g) - no significant differences among treatments.							
Mean	1.7	1.5	2.4	2.0	2.3	2.0	2.5
SD	0.9	0.8	1.7	0.9	1.0	0.8	1.6
Delta mass (g)							
Mean	0.0	-0.1	-0.1	-0.1	-0.1	-0.1	-0.2
Initial Length (cm) - no significant differences among treatments.							
Mean	3.9	3.9	3.9	3.8	3.8	3.8	3.8
SD	0.4	0.2	0.1	0.4	0.2	0.3	0.2
Final length (cm) - no significant differences among treatments.							
Mean	3.9	3.9	3.9	3.8	3.8	3.9	3.8
SD	0.4	0.2	0.1	0.4	0.2	0.1	0.2
Delta length (cm)							
Mean	0.0	0.0	0.0	0.0	0.1	0.1	0.0

* data analyzed by the Saskatchewan Research Council (SRC) from water collected at the study's end

Table 2-4. Water quality data (mean + 1 SD) collected during the four-day pike exposure conducted in the summer of 2009 at the University of Saskatchewan (all comparisons tested at alpha of 0.05)

Concentrations (ppm)							
Target total dissolved solids	**250**	**1000**	**2000**	**4000**	**6000**	**8000**	**16000**
Actual total dissolved solids*	270	990	2070	4130	6070	8000	17600
Temperature (C) – all treatments significantly different.							
Mean	22.3	21.9	21.6	20.9	22.8	22.4	22.1
SD	0.3	0.4	0.7	0.1	0.2	0.4	0.3
Dissolved oxygen (%) – no significant differences among treatments.							
Mean	90.9	91.0	91.1	91.2	90.6	91.0	91.6
SD	1.0	0.7	1.1	0.8	0.8	0.8	0.8
pH (pH units) – all comparisons differ except 8000 and 16000.							
Mean	7.9	8.3	8.5	8.7	8.7	8.8	8.8
SD	0.02	0.01	0.03	0.02	0.03	0.07	0.1
Conductivity (uS/cm) – all treatments significantly different.							
Mean	417.97	1304.6	2431.2	4181.5	6250.0	7809.1	15564.8
SD	11.6	33.6	97.0	85.7	204.4	147.6	373.5
Total dissolved solids (ppm)** – all treatments significantly different.							
Mean	295.3	929.1	1740.8	3038.5	4369.1	5501.8	11041.7
SD	8.3	27.2	59.5	62.3	133.8	93.9	308.5

* data analyzed by the Saskatchewan Research Council (SRC) from water collected at the study's end

** TDS collected by YSI 85 (note: values are derived from conductivity assuming all ions are NaCl and do not represent true TDS)

Table 2-5. Water quality data (mean + 1 SD) collected during the four-day walleye exposure conducted in the summer of 2009 at the University of Saskatchewan (all comparisons tested at alpha of 0.05)

Concentrations (ppm)							
Target total dissolved solids	**250**	**1000**	**2000**	**4000**	**6000**	**8000**	**16000**
Actual total dissolved solids*	213	938	2030	3860	5560	7530	15400
Temperature (C) – 250 and 1000 different from all, 6000 different from 8000 and 16000, 8000 different from 2000 and 16000.							
Mean	23.0	22.5	21.7	21.1	23.1	22.6	22.4
SD	0.4	0.5	0.8	0.2	0.2	0.3	0.2
Dissolved oxygen (%) – no significant differences among treatments.							
Mean	93.1	93.5	94.0	94.1	93.7	94.0	94.1
SD	0.4	0.5	0.3	0.3	0.3	0.4	0.3
pH (pH units) – all comparisons differ except 8000 and 16000 differ significantly.							
Mean	8.0	8.4	8.6	8.8	8.9	8.9	8.8
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Conductivity (uS/cm) – all treatments significantly different.							
Mean	345.2	1257.8	2384.4	3976.6	5650.7	7231.2	12885.9
SD	3.9	13.5	43.8	66.0	194.6	178.1	137.6
Total dissolved solids (ppm) –** all treatments significantly different.							
Mean	240.5	885.3	1706.0	2877.6	3933.0	5072.2	9088.3
SD	2.8	11.0	27.0	56.9	139.7	120.7	87.6

* data analyzed by the Saskatchewan Research Council (SRC) from water collected at the study's end

** TDS collected by YSI 85 (note: values are derived from conductivity assuming all ions are NaCl and do not represent true TDS)

Table 2-6. Water quality data (mean + 1 SD) collected during the four-day fathead minnow exposure conducted in the summer of 2009 at the University of Saskatchewan (all comparisons tested at alpha of 0.05)

Concentrations (ppm)							
Target total dissolved solids	**250**	**1000**	**2000**	**4000**	**6000**	**8000**	**16000**
Actual total dissolved solids*	281	1030	2030	4120	5970	7980	16100
Temperature (C) – all treatments significantly different.							
Mean	22.1	21.7	20.8	20.1	22.6	22.0	21.9
SD	0.3	0.5	1.0	0.2	0.4	0.3	0.5
Dissolved oxygen (%) – no significant differences among treatments.							
Mean	91.5	92.0	93.0	92.9	92.3	92.6	92.2
SD	1.2	0.5	0.6	0.5	0.6	0.3	1.0
pH (pH units) – all comparisons differ except 8000 and 16000 differ significantly.							
Mean	8.1	8.5	8.7	8.9	8.8	8.8	8.6
SD	0.1	0.01	0.01	0.01	0.1	0.01	0.2
Conductivity (uS/cm) – all treatments significantly different.							
Mean	284.3	944.2	1663.8	3016.7	4216.8	5320.7	9411.8
SD	16.5	32.8	54.7	118.2	100.9	144.0	193.8
Total dissolved solids (ppm) –** all treatments significantly different.							
Mean	275	929.1	1740.8	3038.5	4369.1	5501.8	11041.7
SD	8.3	27.2	59.5	62.3	133.8	93.9	308.5

* data analyzed by the Saskatchewan Research Council (SRC) from water collected at the study's end

** TDS collected by YSI 85 (note: values are derived from conductivity assuming all ions are NaCl and do not represent true TDS)

2.4.1.2 Water Quality

To verify that water quality parameters did not differ among treatments, and to ensure poor water quality did not confound the results, temperature, dissolved oxygen, pH, conductivity, and TDS were recorded and compared among treatments (Table 2-4,Table 2-5, and Table 2-6). In the pike study, as predicted, significant differences existed between all treatments for both conductivity (Kruskal-Wallis, $H_{(6)} = 67.61$, $p < 0.001$) and TDS ($H_{(6)} = 67.61$, $p < 0.001$). Analysis of dissolved oxygen resulted in no significant differences among treatments ($H_{(6)} = 9.45$, $p = 0.15$). However, both pH and temp displayed some significant differences among treatments ($H_{(6)} = 60.73$, $p < 0.001$, $H_{(6)} = 47.88$, $p < 0.001$, respectively, see Table 2-4 for paired comparisons)

Similar to the pike study, the walleye study had significant differences in the TDS and conductivity measurements among all treatments (Kruskal-Wallis, $H_{(6)} = 67.61$, $p < 0.001$, $H_{(6)} = 67.61$, $p < 0.001$, respectively). Significant differences were also found in dissolved oxygen ($H_{(6)} = 32.72$, $p < 0.001$), pH ($H_{(6)} = 60.47$, $p < 0.001$) and temperature ($H_{(6)} = 48.11$, $p < 0.001$) (see Table 2-5 for paired comparisons).

As in the previous studies, the fathead minnow exposure had significant differences in the TDS and conductivity measurements among all treatments (Kruskal-Wallis, $H_{(6)} = 67.61$, $p < 0.001$, $H_{(6)} = 67.61$, $p < 0.001$, respectively). Significant differences were also found in dissolved oxygen ($H_{(6)} = 24.35$, $p < 0.001$), pH ($H_{(6)} = 51.37$, $p < 0.001$) and temperature ($H_{(6)} = 46.72$, df = 6, $p < 0.001$) (see Table 2-6 for paired comparisons).

To verify that actual TDS values did not differ from predicted values, and to obtain a detailed water chemistry profile, a water sample was collected from each tank and combined into a single sample for each treatment. This process was repeated for all three.

2.4.1.3 Behavioural Responses

Behavioural responses consisted of daily recordings of each fish for a period of five minutes, during which time spent moving was recorded. Results were analyzed using a contrast repeated measures ANOVA, which compares all treatment groups individually to the control. The purpose of using the repeated measures ANOVA in this instance is to increase statistical power by incorporating measurements taken from each fish over a four-day period. The repeated measures ANOVA will measure the impact of the various levels of TDS using the repeated measure to account for a portion of

the variance, thus providing a *p*-value specific to the concentrations. Results indicate significant reductions in the movement of pike exposed to all concentrations of 2000 ppm and greater (Figure 2-1, ANOVA, $F_{(5,54)} = 3.764$, $p = 0.005$, post hoc tests for 2000-8000: $p < 0.05$).

Walleye behaviour was less sensitive to TDS levels, with no significant differences noted in the analysis (Figure 2-2, $F_{(5,54)} = 1.79$, $p = 0.13$). Minnows produced an intermediate response, demonstrating significant behavioural changes in response to 6000 ppm and 8000 ppm (Figure 2-3, $F_{(5,51)} = 2.781$, $p = 0.04$, post hoc tests, $p < 0.05$, all other comparisons: $p > 0.05$).

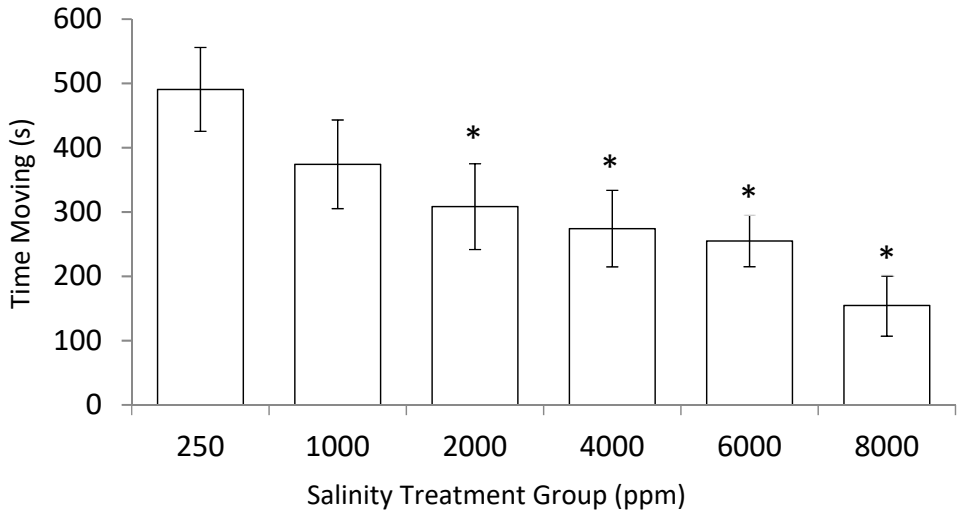

Figure 2-1. Mean (+ SE) number of seconds spent moving (/1200 seconds) by pike over the four-day period. Note: 16000 ppm treatment not shown due to high mortality. *denotes statistically significant difference with control (250 ppm)

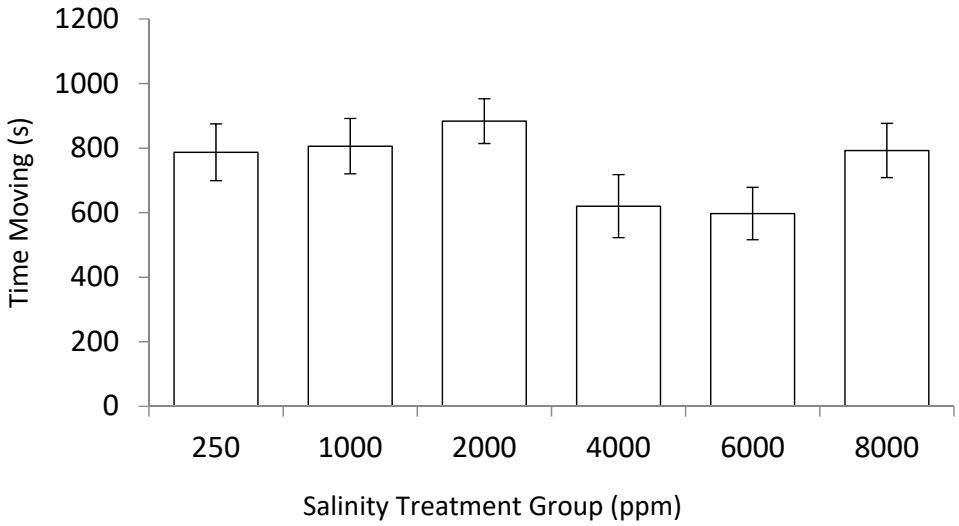

Figure 2-2. Mean (+ SE) number of seconds spent moving (/1200 seconds) by walleye over the four-day period. 16000 ppm treatment not shown due to high mortality. No statistically significant difference was found among treatment groups.

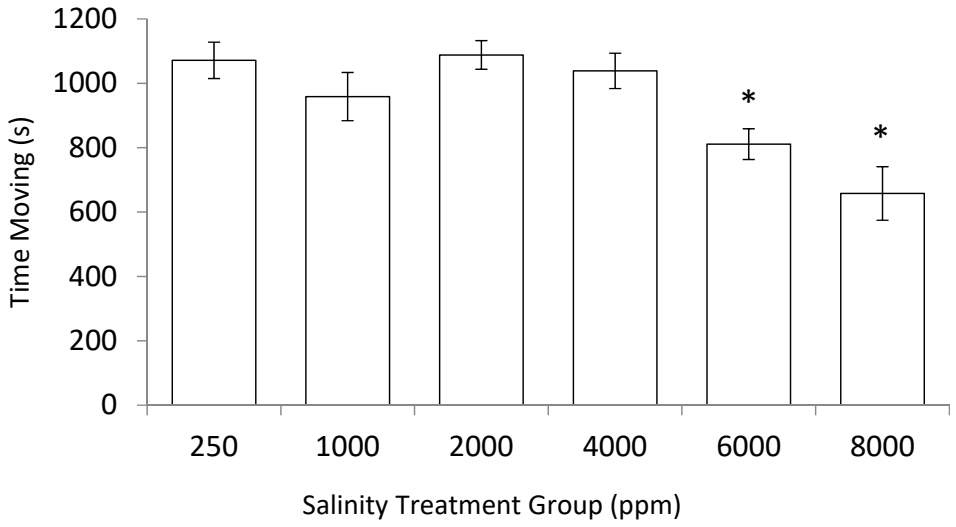

Figure 2-3. Mean (+ SE) number of seconds spent moving (/1200 seconds) by fathead minnows over the four-day period. 16000 ppm treatment not shown due to high mortality. *denotes statistically significant difference with control (250 ppm)

2.4.1.4 LC50 Calculations

Experimental results demonstrated complete mortality of pike in the 16000 ppm treatment over the 96-hour period (see Figure 2-4 for cumulative mortality in the 16000 ppm treatment). All other treatment groups displayed zero mortality. Calculations of the LC50 value used a logit regression model, and arrived at a 96-hour LC50 value of 11627 ppm. The 95% confidence limits for the LC50 value are relatively broad (7544 - 28370 ppm, Figure 2-5) due to the fact that mortality went from zero to 100% with no treatments showing a median response.

Analysis conducted in an identical fashion resulted in a walleye LC50 value of 8316 ppm (95% CI: 7305 - 17353 ppm; see Figure 2-6 and Figure 2-7).

Analysis of the fathead minnow exposure data resulted in an LC50 value of 11627 ppm (95% CI: 7544 - 28370 ppm; see Figure 2-8 and Figure 2-9) (note: due to identical cumulative mortality at 96 hours, pike and minnows shared identical values).

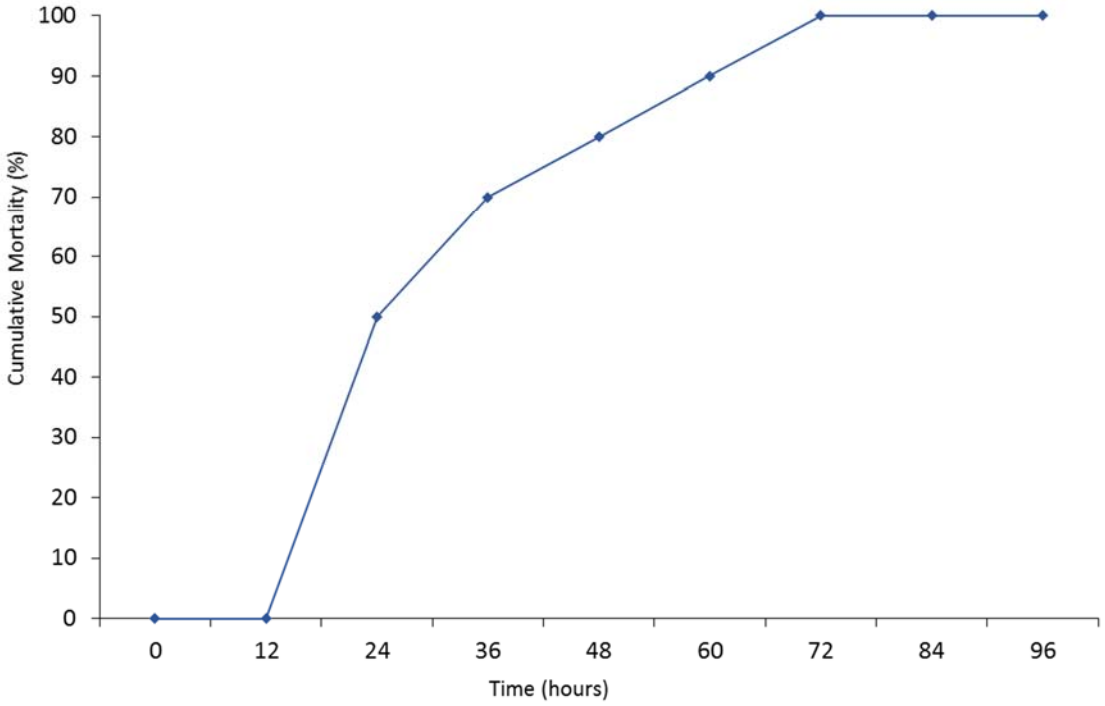

Figure 2-4. Cumulative mortality of pike exposed to 16000 ppm TDS over a 96-hour period.

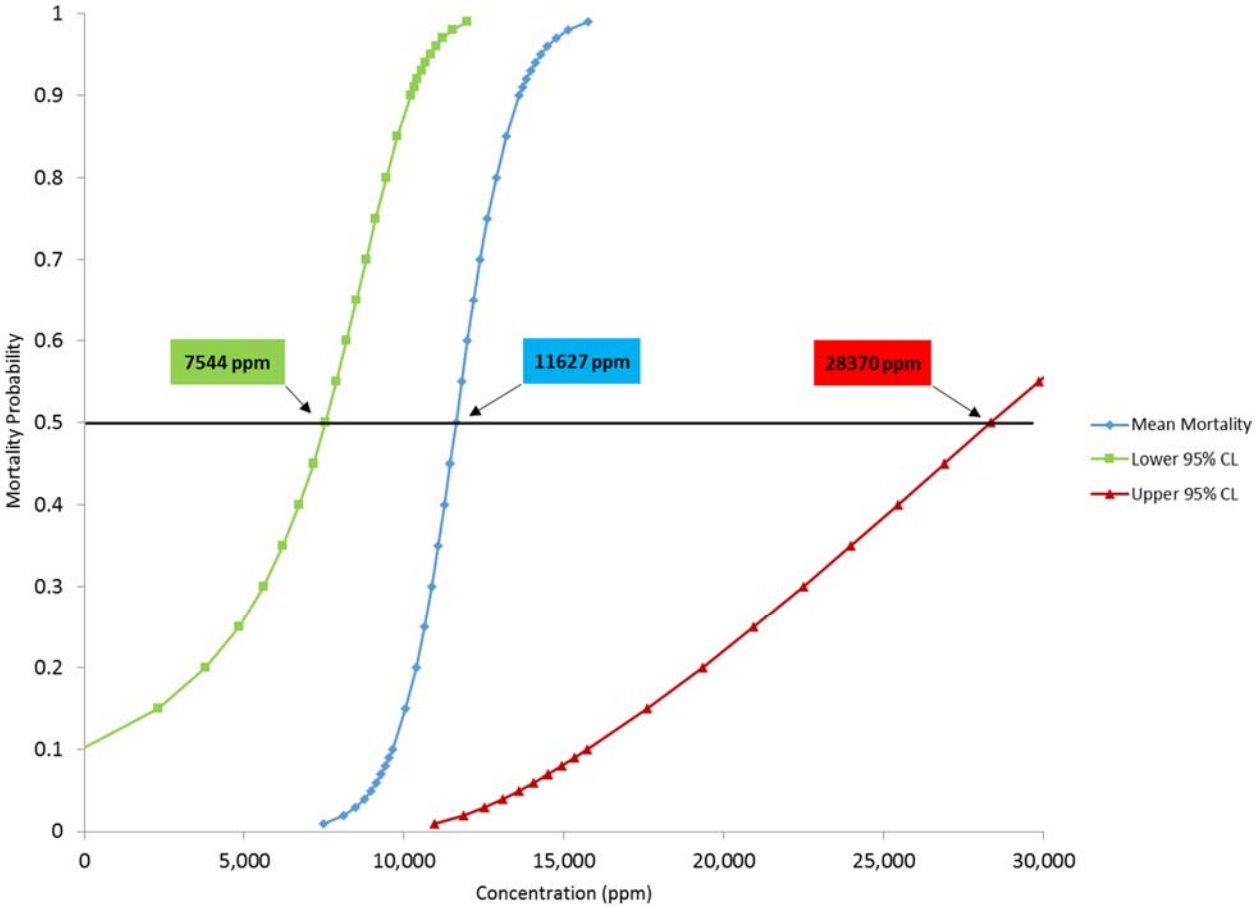

Figure 2-5. Logit 96-hour LC50 calculation for juvenile pike exposed to total dissolved solids. Note: Blue box denotes LC50, green box denotes lower 95% confidence limit of LC50, and red box denotes upper 95% confidence limit of LC50.

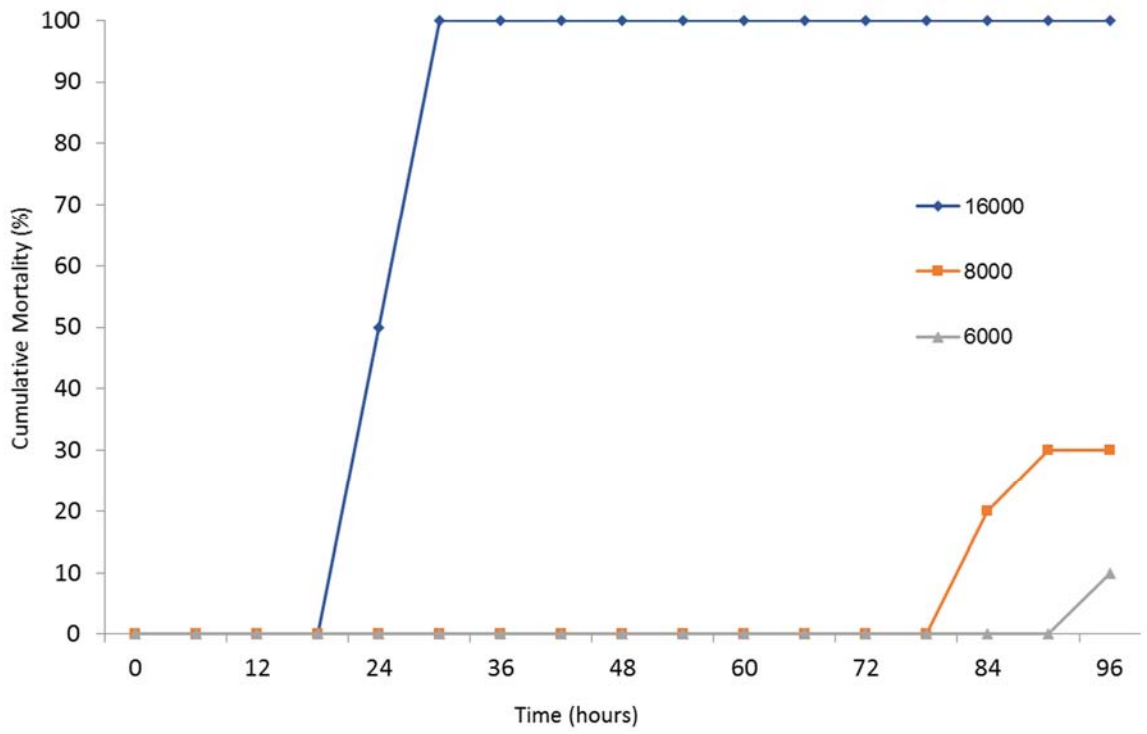

Figure 2-6. Cumulative mortality of walleye exposed to 16000 ppm TDS over a 96-hour period.

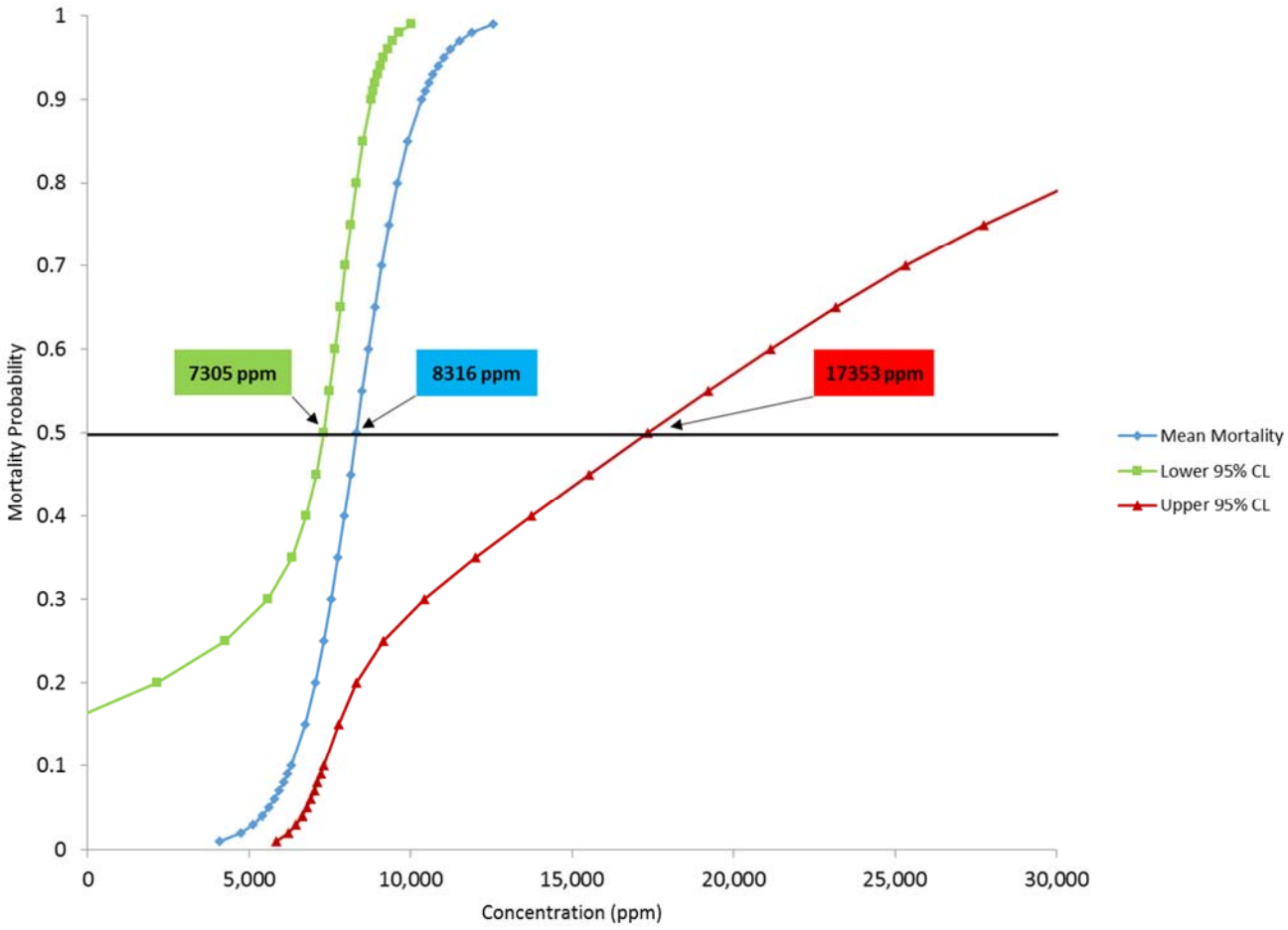

Figure 2-7. Logit 96-hour LC50 calculation for juvenile walleye exposed to total dissolved solids. Note: Blue box denotes LC50, green box denotes lower 95% confidence limit of LC50, and red box denotes upper 95% confidence limit of LC50.

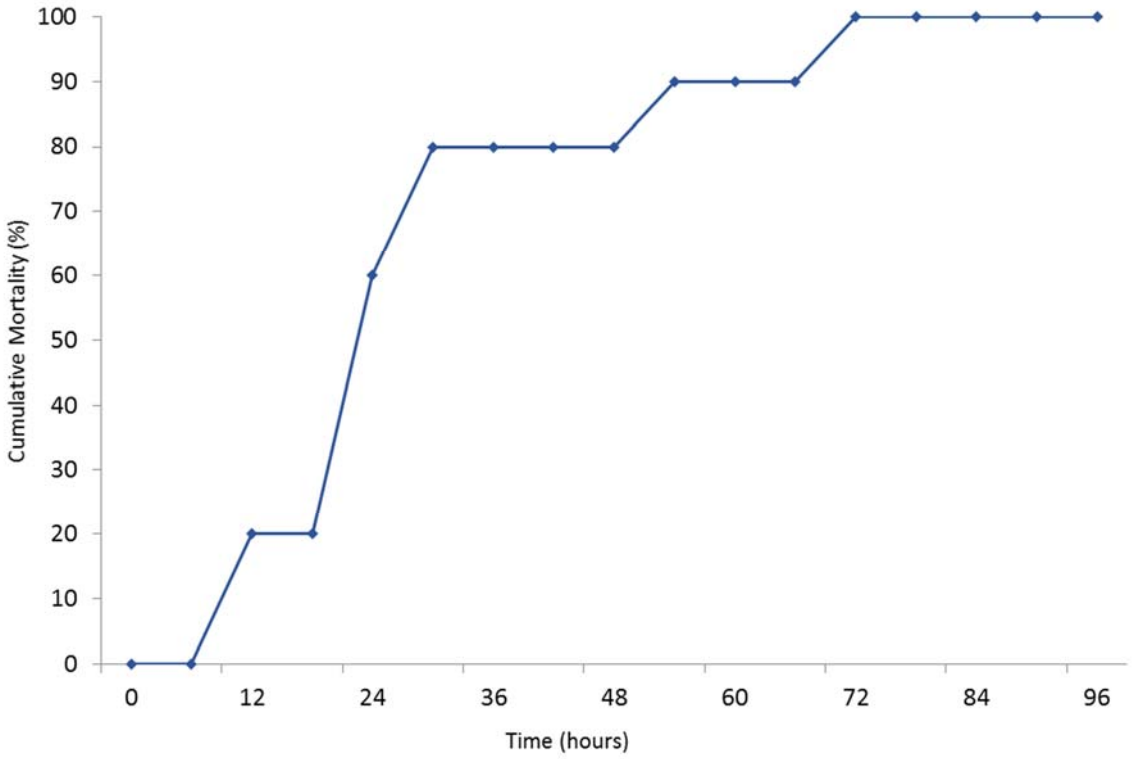

Figure 2-8. Cumulative mortality of fathead minnows exposed to 16000 ppm TDS over a 96-hour period.

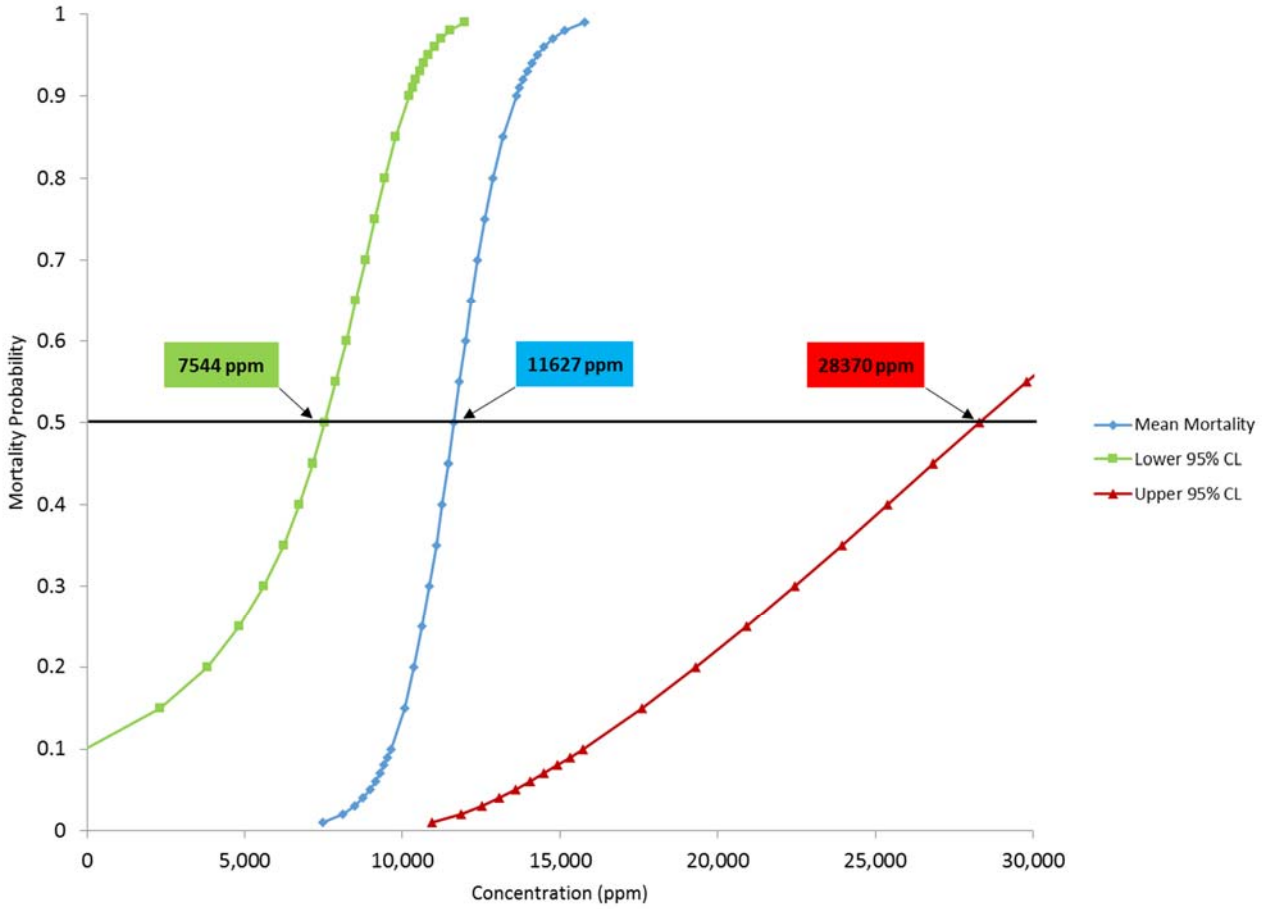

Figure 2-9. Logit 96-hour LC50 calculation for adult fathead minnows exposed to total dissolved solids. Note: Blue box denotes LC50, green box denotes lower 95% confidence limit of LC50, and red box denotes upper 95% confidence limit of LC50.

2.5 Conclusion

With the exception of walleye, behaviour was found to be most sensitive of the two variables in response to TDS. Results of the current study indicate behavioural modification at TDS values as low as 2000 ppm in pike and 6000 ppm in minnows, while walleye behaviour remained unchanged at all levels. However, what remains to be determined is the physiological cause of this decrease in movement and its consequences to the individual. It is recommended that future studies examine the impacts of this decreased movement on feeding and other critical behaviours such as antipredator responses. If it is determined that these activities can be adequately performed, it can be concluded that although a decrease in movement is noted, it is likely to have minimal impact to the individual. If, on the other hand, food or predatory stimulus fails to elicit movement, it could be concluded that the lack of movement could be detrimental to individual health and population stability.

Results of the walleye LC50 were slightly lower than values reported by Rawson and Moore (1944). In their 1944 survey, they regularly found walleye thriving in waters with TDS levels between 10000 and 15000 ppm. This difference may be attributed to the fact that the walleye used in the current study were hesitant to feed, and entered the trial with lower body reserves than their wild counterparts. The LC50 value of 11627 ppm for pike and minnows compares favourably with previous studies (Jacobsen et al., 2007; Rawson & Moore, 1944). The Jacobsen et al. study reported significant pike mortality between 12000-14000 ppm, while Rawson and Moore were unable to collect minnows in saline Saskatchewan water bodies with TDS concentrations higher than 10000 ppm. It should be noted that Jacobsen et al. (2007) used pike approximately half the size of the fish in this study. As previously indicated, size may impact an individual's ability to tolerate TDS (Mojazi, 2009), with smaller fish potentially less able than larger fish to successfully osmoregulate. It should be noted as well that the ion composition would most likely play a significant role in survival, and the Jacobsen study used experimental water with Na^+ and Cl^- as the dominant ions.

In conclusion, given the fact that Lake Lenore has only reached 8000 ppm once in the last 70 years and is currently around 4000 ppm, no imminent or significant mortality is predicted in the pike, walleye, or minnow populations. It should be noted that the fathead minnow served as a surrogate in this study, representing the forage fish community, and is not known to inhabit Lake Lenore. Several attempts by Fisheries and Oceans Canada to collect forage species for the current study resulted in limited catch. Catches consisted of limited numbers of Iowa Darter (*Etheostoma exile*), ninespine stickleback (*Pungitius pungitius*) and brook stickleback (*Culaea inconstans*).

Chapter 3: Study 2—Impact of salinity on fathead minnow (*Pimephales promelas*) antipredator responses

This study was published in Chemosphere. The published version has been altered to match the formatting of this thesis.

Hoover, Z., Ferrari, M. C. O., & Chivers, D. P. (2013). The effects of sub-lethal salinity concentrations on the anti-predator responses of fathead minnows. *Chemosphere, 90*(3), 1047-1052.

3.1 Abstract

Salinization, both natural and anthropogenic, of inland waters is a major facet of environmental change, and can have detrimental effects on aquatic systems. Fish facing increasing levels of salinity must do more than simply survive salinization, they must also undertake important behaviours such as predator avoidance. Here, we exposed fathead minnows (*Pimephales promelas*) to three levels of salinity crossed by three levels of predation risk cues. We found a reduction in pre-stimulus movement and a lowered intensity of antipredator response for the highest salinity exposure (8000 ppm). We also found that the typical threat-sensitive antipredator response (an important behaviour conferring fitness advantages) was absent in the two highest salinity exposure treatments. Our data demonstrate that salinization can have negative effects on critical behaviours well below physiological tolerance levels.

3.2 Introduction

Environmental change can dramatically affect aquatic ecosystems. Climatic events such as drought can alter water temperature, dissolved oxygen, salinity, and the concentration of dissolved nutrients. These changes have the potential to stress organisms, and possibly entire aquatic ecosystems, beyond the point of recovery (Bond, Lake, & Arthington, 2008). Additionally, humans can exacerbate the effects of drought through practices such as water diversion and crop irrigation (Lake, 2011). However, aquatic systems tend to be resilient, and can often cope with environmental change if the rate of change is not excessive (Flower, 2001). Unfortunately, the rate of change associated with many human activities tends to be high, and the sheer number of ways in which humans can modify the environment is staggering. Anthropogenic environmental change also tends to be multi-faceted; aquatic ecosystems are often affected by more than one stressor at a time. In short, aquatic ecosystems face a number of challenges, and are affected by a variety of natural and anthropogenic changes.

Salinization, the increase of salinity in water bodies, is one such change facing many inland waters. Salinization can occur in two ways. Natural, or primary salinization, has no anthropogenic basis. It is typically caused by the accumulation and concentration of salts via ice-cover, weathering of rocks and soil, and the process of evaporation and subsequent concentration of salts. In contrast, secondary salinization is caused by human activities, and may be more acute. Secondary salinization can have many causes, including: clearing of natural vegetation for development, discharge of wastewater, irrigation, runoff, dams, and mining activities (Williams, 2001). Confounding both primary and secondary

salinization, global climate change almost certainly plays a role, but is notoriously difficult to discern (Covich et al., 1997; Pratchett et al., 2011).

Increasing salinity can affect aquatic ecosystems in many ways. It can cause shifts in biotic communities, limit biodiversity, exclude less tolerant species, and cause acute or chronic effects at specific life stages (Weber-Scannell & Duffy, 2007). Additionally, salinity may increase the toxicity of other pollutants in the aquatic environment (Noyes et al., 2009). Salinization of inland waters represents a serious threat to ecosystems and humans alike. If left unchecked, increasing salinity could leave many inland water bodies unfit for animal and/or human uses (Williams, 1987).

The physiological effects of increasing salinity have been well studied in aquatic organisms. However, many studies have focused on NaCl (for example, Kefford, Papas, Metzeling, & Nugegoda, 2004; Luz et al., 2008). This bias in previous research is a concern because other major ions, common to inland waters around the world (such as MgSO4 dominated lakes in Saskatchewan), may have even more dramatic effects than NaCl. For instance, Mount et al. (1997) found that the 96-h LC50 for fathead minnows (*Pimephales promelas*) varied from <510 to 7960 parts per million (ppm) depending on the ion ratio and salts present in the experimental water, with the following relative ion toxicity: $K^+ > HCO_3^- \approx Mg^{2+} > Cl^- > SO_4^{2-}$. Similarly, for rainbow trout (*Oncorhynchus mykiss*) and larval chironomids (*Chironomus tentans*), Chapman et al. (2000) found the toxicity of mining effluents was not predictable from total dissolved solids (TDS) concentration alone, but instead depended on the specific combination and concentration of ions.

In addition to overcoming the physiological stress imposed by salinity, aquatic animals must successfully forage, mate, and avoid predators while exposed to these stressors. The goal of our current work was to understand how an increase in salinity influences the ability of a prey fish (fathead minnows) to respond to predation risk, given that a combination of chemical and biological stressors have been shown to interact synergistically to influence the behaviour of aquatic animals (Relyea & Mills, 2001).

Fathead minnows are small bodied prey fish common throughout much of central North America and as such, are often subjected to both primary and secondary salinization. Minnows have well characterized antipredator behaviours and show a remarkable ability to distinguish among a variety of chemical cues indicating risk. For example, these fish can determine the size, density, and proximity of predators based on the odour signature of their predators (Ferrari et al., 2010). Minnows also respond

to chemical alarm cues released by nearby conspecifics which have been attacked by a predator (Chivers & Smith, 1998). These cues serve as an early warning, alerting prey fish to the presence of a nearby predator.

Ferrari et al. (2005) showed that minnows exhibit threat-sensitive responses to chemical alarm cues. The minnows increase the intensity of their antipredator response (an increase in shelter use and a reduction in activity) when exposed to increasing concentrations of alarm cues. Essentially, fathead minnows modulate their antipredator response to match the perceived level of threat, based on the concentration of alarm cues present (Helfman, 1989). This confers a fitness advantage: time and resources are not wasted due to excessive or insufficient antipredator behaviours (Lima & Dill, 1990).

Here, we tested whether salinity influenced the ability of fathead minnows to exhibit threat-sensitive antipredator behaviour by exposing them to three sub-lethal concentrations of salinity and measuring their antipredator response to three risk levels: no risk (water), low, or high risk (low or high concentration of alarm cues).

3.3 Methods

3.3.1 Experimental design

Using a completely randomized 3x3 design, we tested the effects of three salinity levels (1000 ppm, 4000 ppm, or 8000 ppm) and three predation risk cues (water, low, or high concentration of alarm cues) on the antipredator responses of fathead minnows (N = 180 fish, 20 per treatment). After an acclimation period to their respective salinity levels, the fish were exposed to one of three cues and their antipredator response was recorded.

3.3.2 Experimental fish

Adult fathead minnows were captured from Feedlot Pond (salinity 300 ppm), located on the University of Saskatchewan campus, in November 2009 and housed in the R.J.F. Smith Center for Aquatic Ecology in a 3500 L flow through tank (filled with dechlorinated tap water, salinity 300 ppm). They were fed commercial fish flakes (Nutrafin Max Flake Food, Rolf C. Hagen Inc., Montreal, QC) *ad libitum* and held at room temperature with a 16:8 h light:dark photoperiod and at least 80% oxygen

saturation. This experiment took place in the spring of 2010, prior to the breeding season of the minnows.

3.3.3 Stimulus collection

Alarm cues were prepared using 15 fathead minnows (mean ± SD: fork length 5.5 ± 1.3 cm; weight 1.9 ± 1.2 g) following the method described in (Ferrari et al., 2005). The minnows were euthanized by cervical dislocation, in accordance with University of Saskatchewan Animal Care protocol #20100023. Skin fillets were removed from each side of the body and immediately placed in 100 ml of chilled distilled water. The skin solution was then homogenized and filtered through glass wool. This procedure resulted in 42.9 cm^2 of skin in 858.4 ml of distilled water to give a stock solution of 1 cm^2 of skin per 20 ml. This stock solution was then serially diluted to obtain high (1 cm^2/40 L) and low (1 cm^2/ 80 L) concentration alarm cue solutions. These solutions, along with distilled water, were frozen in 20 ml aliquots at -20°C until use.

3.3.4 Salinity preparation

Experimental water was prepared by reconstituting reverse osmosis water with sodium carbonate (Na_2CO_3; 1000, 4000, 8000 ppm treatments: 0.181 g, 0.722 g, 1.444 g), potassium chloride (KCl; 0.415 g, 1.661 g, 3.323 g), sodium bicarbonate ($NaHCO_3$; 1.261 g, 5.045 g, 10.091 g), magnesium sulfate ($MgSO_4$; 5.279 g, 21.117 g, 42.234 g), calcium sulfate dihydrate ($CaSO_4 * 2H_2O$; 0.512 g, 2.047 g, 4.093 g), calcium chloride dihydrate ($CaCl_2 * 2H_2O$; 0.074 g, 0.296 g, 0.592 g), and sodium sulfate (Na_2SO_4; 1.403 g, 5.613 g, 11.225 g). All chemicals were American Chemical Society (ACS) reagent grade or higher, and were chosen to mimic the ion ratio of Lake Lenore—a typical sulfate-dominated saline lake in Saskatchewan, Canada. See Figure 3-1 for milligram equivalent per liter (mEq/L) ion composition. Due to the absence of published fathead minnow toxicity data for sulfate dominated water bodies, sub-lethal salinity concentrations were chosen based on the natural distribution of fathead minnows in these systems (maximum 10000 ppm, Rawson & Moore, 1944). Although the test fish were collected from a pond with a salinity of ≈ 300 ppm and maintained in the laboratory for several months at a salinity ≈ 250 ppm (see above), we chose 1000 ppm as our lowest salinity concentration because preliminary

experiments revealed that the behaviour (activity level, etc.) of the fish were not influenced at this salinity level.

Salinity levels were increased by removing 500 ml of water from each 9 L experimental tank, pooling the removed portions for each treatment level in a separate mixing container, adding salts while stirring with a hand mixer, and returning the removed portion to each tank. This approach ensured that the salts were completely dissolved in solution before they were added to experimental tanks, and helped minimize the precipitation problems associated with creating a single stock solution.

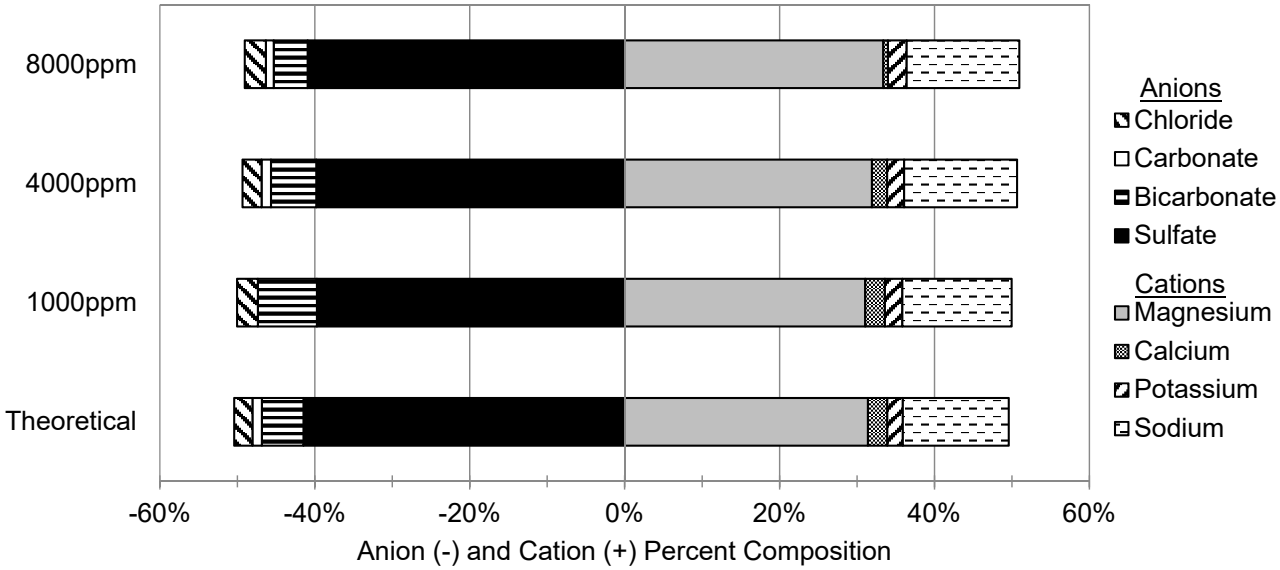

Figure 3-1. Average milligram equivalent per liter ion composition for theoretical and treatment waters. Theoretical values are based on sampling of Lake Lenore by the Saskatchewan Watershed Authority (2007–2008). Presented mEq/L values are averages of the three alarm cue treatments for each salinity level, based on the results of independent laboratory analysis. Ions are divided into anions and cations. Note that percent composition should be considered an absolute value; anions are represented by negative numbers to facilitate comparison.

3.3.5 Test apparatus and acclimation period

A single minnow was placed in each experimental tank (N = 180). Experimental tanks consisted of 9 L plastic aquaria equipped with an air stone, a stimulus injection tube, a lid, and a shelter object (11 x 11 cm ceramic tile with 3 cm legs). After 24 h, salinity was increased over 2 d to experimental levels using the procedure outlined in Section 3.3.4 *Salinity preparation*. Salinity was increased slowly to decrease stress to fish (Kefford et al., 2004; Whiterod & Walker, 2006). Once experimental salinity levels were reached, the fish were given an additional 72 h to acclimate to the new environmental conditions.

3.3.6 Testing procedure

Testing followed well-established protocols (Pollock, Pollock, & Chivers, 2006). Following the acclimation period, fish were exposed to one of three risk cues and their antipredator responses were measured for 8 min prior to and 8 min following the injection of cues into the test tank. During each of these observation periods, we recorded the time (s) that minnows spent moving (*i.e.* actively swimming and/or foraging) and the time (s) spent under shelter (at least 75% of body under shelter). Typical minnow antipredator behaviours include a reduction in movement and an increase in shelter use from the pre-stimulus baseline; these behaviours render minnows less conspicuous to predators, and tend to increase survival (Mathis & Smith, 1993). Tanks were observed (observer was blind to treatment group) between 10:00 and 16:00 in a random order to prevent bias due to order effects, and the same number of tanks from each treatment was observed each day. The observer sat two meters from the tanks and the room was darkened to reduce fright responses attributable to the presence of the observer. To ensure the injection tube was clear of any stagnant water, 60 ml of tank water was withdrawn from the tube and discarded just prior to the start of a trial. An additional 60 ml of tank water was then withdrawn and retained to later flush the stimulus into the tank. After the 8-min pre-stimulus injection period, 2.5 ml of the high or low concentration alarm cue solution or distilled water was injected into the tube and flushed into the tank with the retained tank water. Fish were then monitored for an additional 8 min. At the conclusion of the trial, water quality parameters (conductivity, pH, dissolved oxygen, and temperature) were recorded for each tank with a YSI probe (Professional Plus, YSI Inc., Yellow Springs, OH). Additionally, 25 ml of tank water was withdrawn from each tank and combined by treatment group for analysis by an independent laboratory (Saskatchewan Research Council, Saskatoon, Canada) for verification of ion composition and TDS concentration (Figure 3-1, Table 3-1).

Table 3-1. Mean (± SD) water quality parameters. Percent error for TDS concentration is based on comparison with nominal theoretical values. The negative value for the 8,000 ppm group denotes the slightly lower than theoretical value.

Treatment Group	1000	4000	8000
Conductivity (µS/cm)	1464 (1)	4257 (11)	7610 (29)
Dissolved Oxygen (%)	87.3 (2.3)	87.4 (1.8)	87.9 (2.2)
pH (pH units)	8.3 (0.1)	8.7 (0.1)	8.6 (0.1)
Temperature (°C)	23.9 (1.1)	23.7 (1.1)	23.5 (1.1)
TDS (ppm)	1067	4037	7783
% Error	7%	1%	-3%

3.3.7 Statistical analyses

Statistical analyses were performed with R version 2.13.2 (R Development Core Team, 2011). All data were checked for conformance to test assumptions, and were transformed as noted below when those assumptions were not met.

3.3.7.1 Fish characteristics and water quality

To ensure there were no confounding differences among treatment groups due to size differences, weight and length measurements of each fish were taken before tank assignment. Size and weight of test fish were compared between salinity and alarm cue treatments using a 3x3 MANOVA. Water quality parameters (conductivity, pH, dissolved oxygen, and temperature) were compared among the three salinity levels using a 1-way MANOVA. ANOVAs were then performed to investigate which of the parameters differed significantly among levels. Significant differences were predicted for conductivity and pH, as conductivity increases with increasing salinity and pH tends to increase as well. Saline waters (salinity >3000 ppm, Williams, 1964) tend to have higher pH values than freshwaters because 1) the salts in saline waters are involved in acid–base interactions in the water, and 2) increased

alkalinity (due to increased concentrations of HCO_3 and CO^2_3) raises the equilibrium pH of saline water (Hinga, 2002; Morel & Hering, 1993).

3.3.7.2 Behavioural data

Data for pre-stimulus movement were heteroscedastic and were not normally distributed. Therefore, the data were square root transformed. A 1-way ANOVA was conducted to determine any effect of salinity on baseline activity among treatment groups.

Due to differences in pre-stimulus movement, percent change in movement (post-stimulus–pre-stimulus/pre-stimulus) was used as a measure of response to alarm cues. Percent change in movement was not normally distributed. Because this measure is an unbounded percentage with both positive and negative numbers, data were rank transformed. A 3x3 ANOVA was then performed to determine any differences among treatment groups.

To determine if salinity affected overall intensity of response, a 1-way ANOVA was performed on percent change in movement for fish exposed to alarm cues (excluding fish exposed to water), followed by Tukey's HSD to evaluate any significant differences. To determine if salinity affected the ability of minnows to respond to risk in a threat-sensitive manner, separate ANOVAs were performed on percent change in movement among alarm cue treatments (within each salinity group), and any significant differences were evaluated with Tukey's HSD.

Examination of the means for pre-stimulus shelter use revealed that minnows spent a large percentage of time under shelter (mean ± SE: 1000 ppm 78 ± 5%; 4000 ppm 72 ± 5%; 8000 ppm 84 ± 4%). Shelter use is a measure of risk avoidance. Therefore, sheltering behaviour should be rare in the absence of risk. Because minnows in this experiment spent so much time under shelter before being exposed to alarm cues, shelter use was deemed an inappropriate measure, and subsequently removed from further analyses (see Section 3.5 *Discussion* for details).

3.4 Results

3.4.1 Fish characteristics and water quality

Standard length (mean ± SD: 5.2 ± 0.9 cm) and weight (2.4 ± 1.2 g) did not differ among experimental groups (Pillai's Trace, salinity: $F_{(2,167)} = 0.02$, $p = 0.6$; cue: $F_{(2,167)} = 0.03$, $p = 0.2$; interaction: $F_{(4,167)} = 0.05$, $p = 0.3$). A significant difference in water quality parameters was found among salinity treatment groups (Pillai's Trace: $F_{(2,173)} = 1.7$, $p < 0.001$, Table 3-1). Both conductivity ($F_{(2,173)} = 17876$, $p < 0.001$) and pH ($F_{(2,173)} = 287$, $p < 0.001$) showed significant differences among groups. These differences were only present among salinity groups—no significant differences were found among alarm cue treatments within the same salinity group (conductivity: $F_{(2,173)} = 0.02$, $p = 0.98$; pH: $F_{(2,173)} = 0.04$, $p = 0.96$). Similarly, no difference was found among treatment groups for dissolved oxygen ($F_{(2,173)} = 0.4$, $p = 0.6$) or temperature ($F_{(2,173)} = 0.2$, $p = 0.8$). All TDS values were within 7% of theoretical Lake Lenore values.

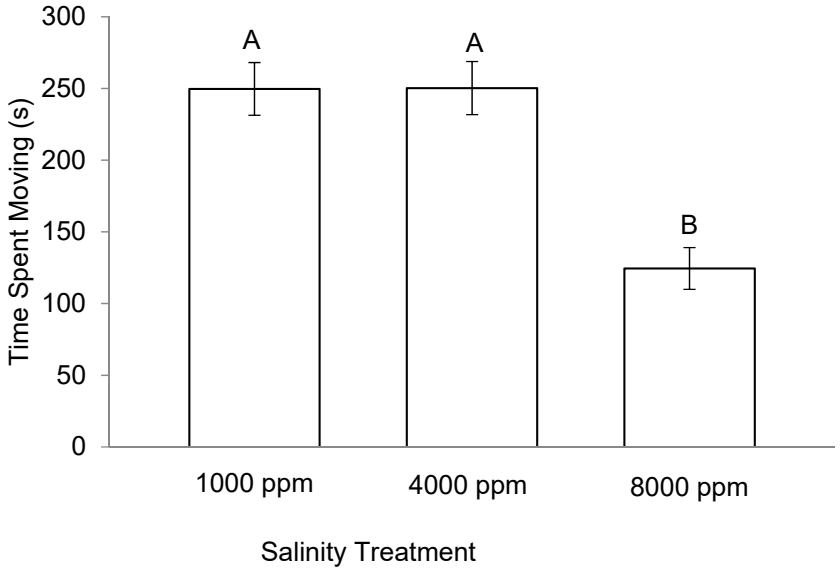

Figure 3-2. Mean (± SE) time spent moving during the 8-min pre-stimulus period, for minnows (n=60 per treatment) maintained in the three different salinities. Different letters above the error bars indicate significant differences at alpha of 0.05.

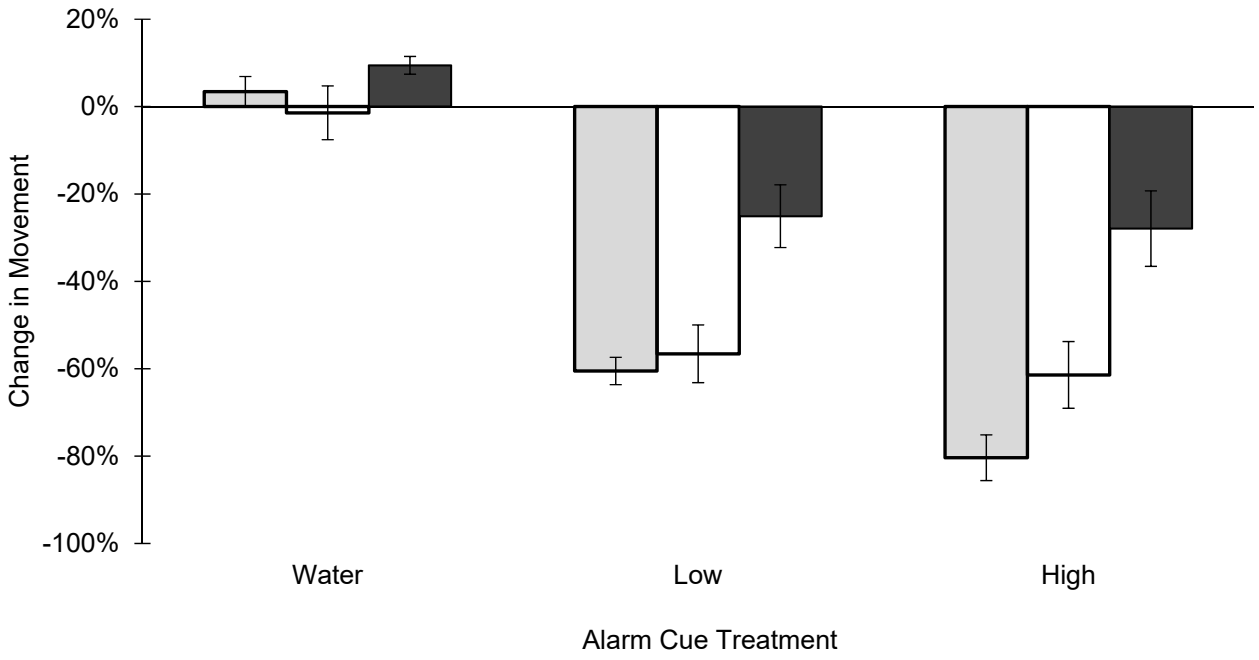

Figure 3-3. Percent change in movement. Mean (± SE) percent change in movement when fathead minnows (n=20 per treatment) were maintained in salinities of 1,000 ppm (grey bars), 4,000 ppm (white bars), or 8,000 ppm (black bars).

3.4.2 Behavioural measures

Pre-stimulus data for time spent moving showed that minnows were significantly affected by salinity treatment ($F_{(2,173)} = 22$, $p < 0.001$; Figure 3-2), with fish in the 8000 ppm treatment displaying a lower baseline activity level than those in lower salinity treatments. Percent change in movement was significantly affected by both cue and salinity levels (salinity x cue: $F_{(8,167)} = 32$, $p < 0.001$; Figure 3-3).

Salinity concentration affected the overall intensity of antipredator responses ($F_{(2,114)} = 21$, $p < 0.001$), with minnows in the 8000 ppm treatment group displaying lower intensity antipredator responses than minnows in other treatments (Tukey comparisons: $p < 0.001$).

Minnows in the 1000 ppm treatment displayed typical threat-sensitive antipredator responses ($F_{(2,55)} = 64$, $p < 0.001$), that is, displayed stronger antipredator responses to increasing concentrations of alarm cues (Tukey comparisons: all $p < 0.005$). Minnows exposed to higher salinity levels, however, failed to show this pattern, and did not respond differently to the high and low concentration of alarm cues (4000 ppm: $F_{(2,56)} = 34$, $p < 0.001$, high vs. low: $p = 0.8$; 8000 ppm: $F_{(2,56)} = 11$, $p < 0.001$, high vs. low: $p = 0.9$). See Figure 3-3.

3.5 Discussion

Our study clearly demonstrates that sub-lethal levels of salinity have the potential to affect the behavioural responses of fathead minnows, in terms of baseline activity level, overall intensity of antipredator response, and threat-sensitivity. We found that fish maintained in 8000 ppm salinity water were less active than fish exposed to lower concentrations. This indicates that such levels provide a significant physiological and homeostatic cost to the fish. Lower activity levels relating to such intoxication have been linked to lower food-anticipatory activity, food intake, and growth (Luz et al., 2008).

Our results also indicate that exposure to salinity can decrease the intensity of response to a general risk cue, such as alarm cues. This can be explained in one of two ways. First, salinity may interact with the olfactory receptors of the fish, or the alarm cue itself, causing a decrease in the detection of cues in the water. A lower detection would lead to a lower antipredator response. Alternatively, the lower antipredator response may be the result of a change in the cost-benefit trade-offs of the fish. The increasing salinity likely causes an increase in metabolic costs (Pistole et al., 2008), which would in turn

force the fish to forage more in order to maintain its energy balance (Fu et al., 2009). Given that the observable behavioural response of the fish is the result of a trade-off between foraging and antipredator behaviour, one would predict that an increase in foraging needs would lead to a decrease in antipredator response for a constant risk level, as demonstrated in Brown and Smith (1996). However, this seems to not be the case, given the low pre-stimulus activity level of the 8000 ppm treatment. It may be that the stress of increased metabolic costs overrides the ability or willingness of the fish to either respond maximally to risk or increase foraging behaviour.

Being able to adjust the intensity of antipredator response to match the level of risk experienced is required for prey to adaptively allocate their energy and time, that is, to maximize fitness-related activities while maintaining costly antipredator responses to a level that would ensure survival. Our results indicate that minnows exposed to moderate and high levels of salinity lose their ability to display such threat-sensitive responses. Whether this is the result of a sensory limitation (salinity interfering with olfaction) or an energetic limitation (increasing energy needs preventing the fish from allocating more time/energy into antipredator responses) is unknown.

A number of anthropogenic stressors have been shown to similarly interfere with the ability of prey fishes to respond to predation risk. For instance, freshwater acidification causes salmonid fishes to not respond to conspecific alarm cues (Leduc, Kelly, & Brown, 2004). Such alterations, in turn, cause deficiencies in alarm cue-mediated behaviours, such as learned predator recognition (Leduc, Ferrari, Kelly, & Brown, 2004) and have potential survival consequences (Leduc et al., 2010). Therefore, these specific costs should also be evaluated for salinity with fathead minnows in order to gain a better understanding of the mechanisms limiting their ability to properly respond to risk.

While it is true that both predators and prey may be affected by increasing salinity, predators tend to be larger-bodied, and there is evidence that larger body size may decrease the intensity of adverse effects associated with increasing salinity (Mojazi Amiri et al., 2009). Additionally, species-specific salinity tolerance may play a role in predator-prey interactions. For instance, Ingersoll et al. (1992) found that NaCl-dominated water was acutely toxic to adult fathead minnows between 8000 and 10000 ppm, while Jacobsen et al. (2007), summarizing previous research, reported NaCl toxicity of 12000–18000 ppm for northern pike (*Esox lucius*), a common minnow predator. Though no studies have been conducted to explicitly evaluate potential predator-prey interactions when both fishes are affected by salinity changes, differential species tolerances may lead to differential survival.

Typical antipredator responses of fathead minnows include a reduction in movement and an increase in shelter use. Our study did not include change in shelter use in the analyses because of high levels of pre-stimulus use. This pre-stimulus sheltering behaviour may be explained by the size of the shelter relative the size of the tank. Shelter use experiments are typically undertaken in tanks with a volume of at least 37 L (for example, Pollock et al., 2006). Though shelters used in this study were roughly the same size as those used in 37 L tanks, the experimental tanks used here had a 9 L volume. Therefore, the shelters occupied a much greater area of the tank, and this may help to explain the high pre-stimulus use. In a larger tank, there is more forage area available than in a 9 L tank. Therefore, it may be that the fish found the shelter a preferable environment to rest of the tank—a location from which they could easily monitor the rest of the forage area without having to leave.

Despite the vast number of physiological studies, very few have evaluated the effects of sub-lethal salinity concentrations on freshwater fish behaviour. The distinction between physiological tolerance and behavioural modification threshold is important because some studies have found changes in behaviour well below lethal levels. For instance, Whiterod and Walker (2006) found an LC50 for common carp (*Cyprinus carpio*) of 13000 ppm with behaviour modification at concentrations as low as 7500 ppm. Similarly, at sub-lethal concentrations, (Alcaraz, Bisazza, & García-Berthou, 2008) found that mosquitofish (*Gambusia holbrooki*) competed less successfully with Mediterranean killifish (*Aphanius fasciatus*) for food, and Luz et al. (2008) found decreased locomotion and feeding behaviour in goldfish (*Carassius auratus*).

Salinization, specifically secondary salinization, has been called the greatest cause of degradation in some aquatic ecosystems (James et al., 2003). This statement can be even more pertinent when secondary salinization compounds primary salinization. Driver and Peden (1977) report that salinity concentrations of permanent saline water bodies in the Great Plains of North America can vary seasonally up to 20%. Therefore, most established populations, particularly those which have successfully experienced such salinity fluctuations in the past, should be able to accommodate mild natural salinization. However, when this natural variation is combined with unpredictable rates of secondary salinization, salinity concentrations may become unbearable, compromising the ability of organisms to undertake important behaviours. Future experiments should consider the effects of such stressors on reproductive behaviours and any generational effects. Additionally, acclimation to increased salinity levels should be investigated. It remains unknown whether minnows would eventually overcome the negative effects of salinity, and if so, the time period involved.

Chapter 4: Study 3—Impact of salinity on fathead minnow (*Pimephales promelas*) reproduction

This study was published in Science of the Total Environment. The published version has been altered to match the formatting of this thesis. Additionally, I added Figures 4-2 and 4-3 to the manuscript, and included a description of the power transformations I performed, summarized in Table 4-2.

Hoover, Z., Weisgerber, J. N., Pollock, M. S., Chivers, D. P., & Ferrari, M. C. O. (2013). Sub-lethal increases in salinity affect reproduction in fathead minnows. *Science of the Total Environment, 463–464*, 334-339.

4.1 Abstract

Salinization poses a threat to many inland aquatic ecosystems, especially in areas where natural processes are compounded by anthropogenic salinization. Though survival can be a challenge for stenohaline freshwater fishes facing increasing salinity, it is important to note that essential and complex activities such as reproduction may be affected well below physiological tolerance limits. Here, we exposed fathead minnows (*Pimephales promelas*) to four levels of salinity in order to assess any impacts on several egg production and behavioural endpoints. We found significant reductions in total eggs produced, percent fertilization, number of spawning days, clutch size, total time males spent in the nest, and duration of nest care events. Our data demonstrate that salinization can have negative effects on critical reproductive endpoints.

4.2 Introduction

Salinization is a stressor facing many inland aquatic ecosystems, and is attributable to two basic sources. Natural, or primary salinization, has no anthropogenic basis. It is typically caused by the accumulation and concentration of salts over time. Sources of primary salinization include the concentration of salts during winter ice-over, weathering of rocks and soil, and the process of evaporation and subsequent concentration when evaporation exceeds precipitation. In contrast, secondary salinization is attributable to human activities, and tends to be more acute. The multitude of secondary salinization sources includes: clearing of natural vegetation for land development, wastewater discharge, irrigation, runoff, water diversion, and mining and industrial activities (Williams, 2001). Unfortunately, global climate change almost certainly plays a role in exacerbating both primary and secondary salinization, but this link is notoriously difficult to discern (Covich et al., 1997; Pratchett et al., 2011).

Salinization can pose a daunting challenge to aquatic ecosystems. It can cause shifts in biotic communities, limit biodiversity, exclude less tolerant species, and cause acute or chronic effects at specific life stages (Weber-Scannell & Duffy, 2007). There is also evidence that salinity can increase the toxicity of some organic pollutants in the environment (Noyes et al., 2009). In such a case, salinization of aquatic systems could be a much larger threat than salinization or the presence of organic pollutants alone. No doubt, salinization represents a serious threat to ecosystems and humans alike. If

left unchecked, increasing salinity could render many inland water bodies unfit for animal and/or human use (Williams, 1987).

There has been an abundance of research on the physiological impacts of salinization on aquatic organisms. However, most research focuses exclusively on NaCl (for example, Bezirci et al., 2012; Pistole et al., 2008). While Na^+ and Cl^- are the major ions found in many salinized water bodies around the world, other regions can be dominated by different ions (such as the $MgSO_4$ and $NaSO_4$ dominated lakes of the Northern Great Plains of North America). This is an important distinction because the ion ratio of water has been shown to have dramatic physiological effects. For instance, Mount et al. (1997) found the 96-h LC50 for fathead minnows (*Pimephales promelas*) ranged from <510 to 7960 parts per million (ppm) based on the ion ratio and salts present in the experimental water. Similarly, for rainbow trout (*Oncorhynchus mykiss*) and larval chironomids (*Chironomus tentans*), Chapman et al. (2000) found that the toxicity of mining effluents was not predictable based on total dissolved solids (TDS) concentration alone, but instead depended upon the combination and concentration of ions in the water.

Beyond survival, aquatic organisms must successfully undertake a multitude of activities, such as foraging, avoiding predators, and reproduction. These important behaviours may be affected at salinity concentrations well below physiological tolerances, and therefore may serve as more sensitive endpoints for understanding the ecological impacts of increasing salinity. However, very few studies have examined the behavioural impacts of sub-lethal salinity concentrations on aquatic organisms. A recent study by Hoover et al. (2013) demonstrated such sub-lethal effects. They found that exposure to increased salinity decreased overall movement, the intensity of antipredator responses, and the ability of fathead minnows to respond to predators in a threat-sensitive manner.

Fathead minnows are small bodied fish common throughout much of central North America. Because they are tolerant of a wide range of water quality characteristics (Ankley & Villeneuve, 2006), fathead minnows can be found in a wide variety of inland aquatic systems, where they serve as important consumers and prey. Due to this widespread distribution, fathead minnows are often subjected to both primary and secondary salinization—especially in the Great Plains, where saline lakes (salinity > 3000 ppm, Williams, 1964) are fairly common. Additionally, due to their relatively short life cycle, small size, and ease of acclimation to the laboratory environment, fathead minnows have become ubiquitous in regulatory testing and research (Ankley & Villeneuve, 2006). Recently, fathead minnows have been used in numerous studies evaluating the effects of endocrine disrupting chemicals. This led to

the development of a formal short-term reproduction assay (OECD, 2009; USEPA, 2002) which can be easily adapted to other chemicals/stressors.

Given that sub-lethal concentrations of salinity have been shown to affect important behaviours in fathead minnows, our current study examined the impacts on several reproductive endpoints (Table 4-1). In short, we tested whether salinity would affect reproductive endpoints by exposing them to one of four sub-lethal salinity concentrations in a short-term reproduction assay. We predicted that salinity concentrations would have a negative impact on common measures of reproductive output, and that any reduction in output would also correspond to a reduction of stereotypic courtship and nest guarding behaviours.

Table 4-1. Measured endpoints. Lowercase letters in brackets refer to the sex of the targeted fish.

Egg-based endpoints	Behavioural endpoints
Total eggs produced	Male approaches female (m)
Percent fertilization	Time spent in nest care (m)
Number of spawning days	Duration of nest care events (m)
Clutch size (number of eggs deposited on an individual breeding substrate)	Time in nest (m)
	Duration of nest time events (m)
	Time in nest (f)
	Duration of nest time events (f)
	Number of spawning attempts (m+f)
	Number of nest defense acts (m)

4.3 Methods

4.3.1 Experimental design

Using a completely randomized and fully blind short-term reproductive assay, we tested the effects of four salinity levels (Control, 1000 ppm, 4000 ppm, and 8000 ppm) on several fathead minnow reproductive endpoints (n = 15 pairs per treatment). The experiment consisted of a pre-exposure phase

(N = 90 pairs, duration = 14 days), followed by an exposure phase (duration = 21 days). The pre-exposure phase established successful breeding pairs based on egg production and fertilization success (Hutchinson et al., 2003; Rickwood et al., 2006). The 60 pairs with the highest production and fertility rates were then randomly assigned to exposure treatments. The exposure phase saw those pairs exposed to experimental salinity levels.

4.3.2 Experimental fish

Adult fathead minnows were purchased from a commercial supplier (Osage Catfisheries Inc., Osage Beach, MO, USA) and housed according to sex in two 530 L flow through tanks filled with dechlorinated tap water (salinity ≈ 300 ppm). The minnows were fed commercial fish flakes (Nutrafin Max Flake Food, Rolf C. Hagen Inc., Montreal, QC, Canada) *ad libitum* at 9:00 and 16:00 daily. Additionally, the 16:00 feeding was supplemented with freeze dried blood worms (Omega One, Omega Sea Ltd., Painesville, OH, USA). Minnows were held at approximately 25°C with a 16:8-hour light:dark photoperiod and at least 75% oxygen saturation. The fish were maintained in these conditions for two weeks prior to the beginning of the experiment in order to ensure their health and acclimation to the laboratory environment.

4.3.3 Salinity preparation

Experimental water was prepared by the addition of sodium carbonate (Na_2CO_3), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), magnesium sulfate ($MgSO_4$), calcium sulfate dihydrate ($CaSO_4{*}2H_2O$), calcium chloride dihydrate ($CaCl_{2*}2H_2O$), and sodium sulfate (Na_2SO_4) to dechlorinated tap water. All chemicals were American Chemical Society (ACS) reagent grade or higher, and were chosen to mimic the ion ratio of Lake Lenore—a typical sulfate-dominated saline lake in Saskatchewan, Canada. See Figure 4-1 for a representation of the milligram equivalent per liter (mEq/L) percent ion composition of the experimental water. Due to the scarcity of published fathead minnow toxicity data for sulfate dominated water bodies, sub-lethal salinity concentrations were chosen based on the natural distribution of fathead minnows in these systems (maximum ≈ 10,000 ppm, Rawson & Moore, 1944). Additionally, Hoover et al. (2013) showed that a similar ion ratio could cause a reduction in minnow antipredator behavior at the same levels.

Fresh experimental water was prepared daily by adding the salts and dechlorinated tap water to mixing tanks (150 L volume). The solutions were then circulated within the mixing tanks overnight. This approach aided in the dissolution of salts, and also aided in bringing each solution to room temperature. Control water followed the same procedures as saline water.

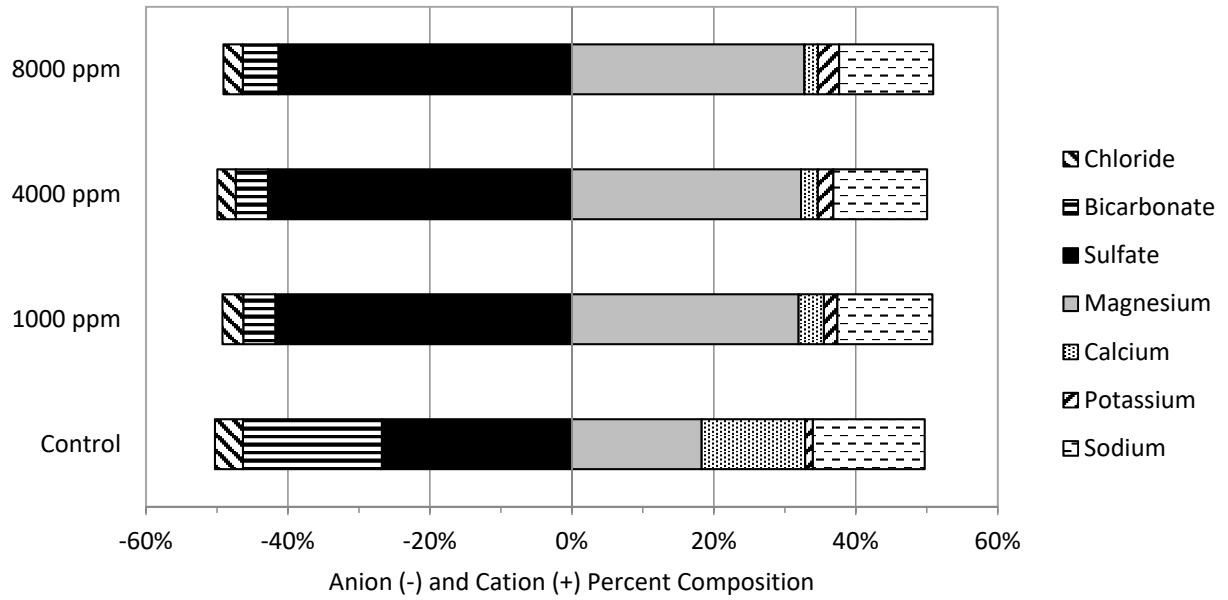

Figure 4-1. Average milligram equivalent per liter ion composition for control and treatment waters. The ion composition of treatment waters is based on sampling of Lake Lenore by the Saskatchewan Watershed Authority (2007–2008). Values are based on the results of independent laboratory analysis (Saskatchewan Research Council, Saskatoon, Canada). Ions are divided into anions and cations. Note that percent composition should be considered an absolute value; anions are represented as negative numbers to facilitate comparison.

4.3.4 Test apparatus and acclimation period

The test apparatus was a semi-static flow through system, designed to facilitate 100% daily water changes. The system consisted of mixing tanks, head tanks for distribution, and experimental tanks which drained to the sewer (See Figure 4-2). Each salinity treatment constituted a separate system; therefore, there were four mixing tanks and four head tanks. Experimental water was prepared and mixed in the mixing tanks. It was then pumped into the head tanks for gravity-fed distribution to individual experimental tanks. Additionally, the head tanks contained overflow pipes which returned water to the mixing tanks in order to maintain consistent head pressure for distribution. Water was distributed to the individual experimental tanks once every afternoon (10 L per tank).

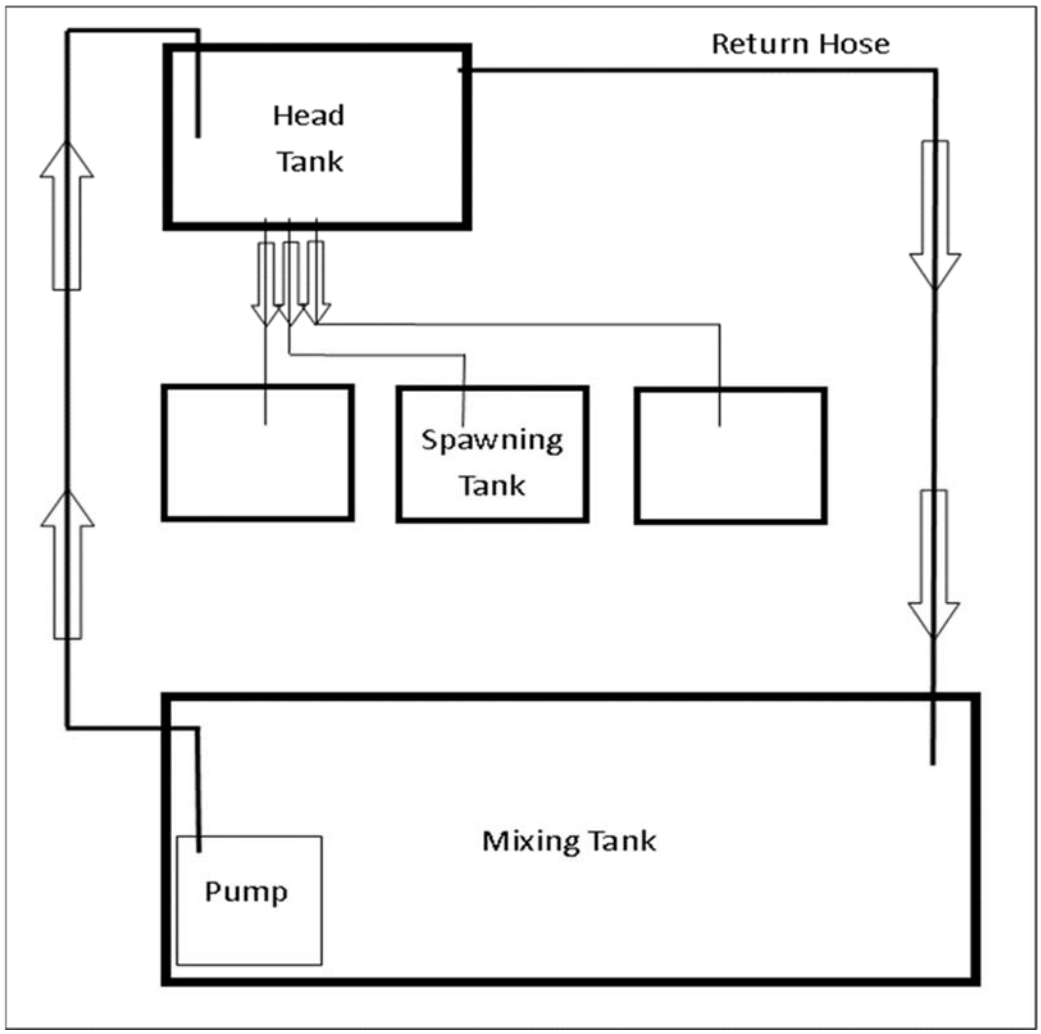

Figure 4-2. Test apparatus setup. Experimental water was produced daily in the mixing tanks. Water was continually circulated from the mixing tanks to the head tanks, and returned via overflow pipes to maintain consistent head pressure during distribution to the spawning tanks. Distribution to spawning tanks was controlled by valves, and 100% water changes were preformed daily, with wastewater drained to the sewer.

Experimental tanks consisted of nine L plastic aquaria with drains. The aquaria were blacked out on three sides to prevent visual cues from adjacent tanks. Each aquarium was equipped with a lid, an air stone, and a breeding substrate (10 cm diameter PVC pipe cut in half, 7.5 cm long). For the pre-exposure phase, the tanks were filled with dechlorinated tap water, and one size-matched pair was placed in each tank. A size-matched (females ≈ 78% the length of males) approach was chosen because, when compared to randomly assigned trios of minnows, this approach has been shown to both reduce the number of animals used and increase breeding efficacy (Pollock et al., 2008). After the pre-exposure phase, and the identification and assignment of successful breeding pairs, salinity was slowly increased over 24 h to experimental levels (two water changes per day, with a higher salinity concentration each time). Salinity was slowly increased in order to minimize stress to the fish (Kefford et al., 2004; Whiterod & Walker, 2006). We wanted to ensure that the minnows in our experiment were free of external disturbances that can reduce reproduction in the laboratory. Consequently, we had a designated experimental room in which lab personnel entered only to feed the test fish, monitor water quality and fish health, and to perform the water changes.

4.3.5 Testing procedure and data collection

Breeding substrates were checked for the presence of eggs at 10:00 am, approximately 30 min after the morning feeding. Those substrates containing eggs were removed and new substrates were provided. The egg bearing substrates were then marked and stored in holding tanks of corresponding salinity. After 24 h, the egg-bearing substrates were removed from the holding tanks and photographed. The images were then assigned a random identification number and analyzed with the Cell Counter plugin of ImageJ (Rasband, 1997-2012). Each photograph was contrast-adjusted until fertilized and infertile eggs could be easily distinguished while zoomed in (as infertile eggs are opaque or clear with a white dot where the yolk has precipitated (Ankley et al., 2001)), and each egg was tagged according to its fertilization state. See Figure 4-3 for an example photograph.

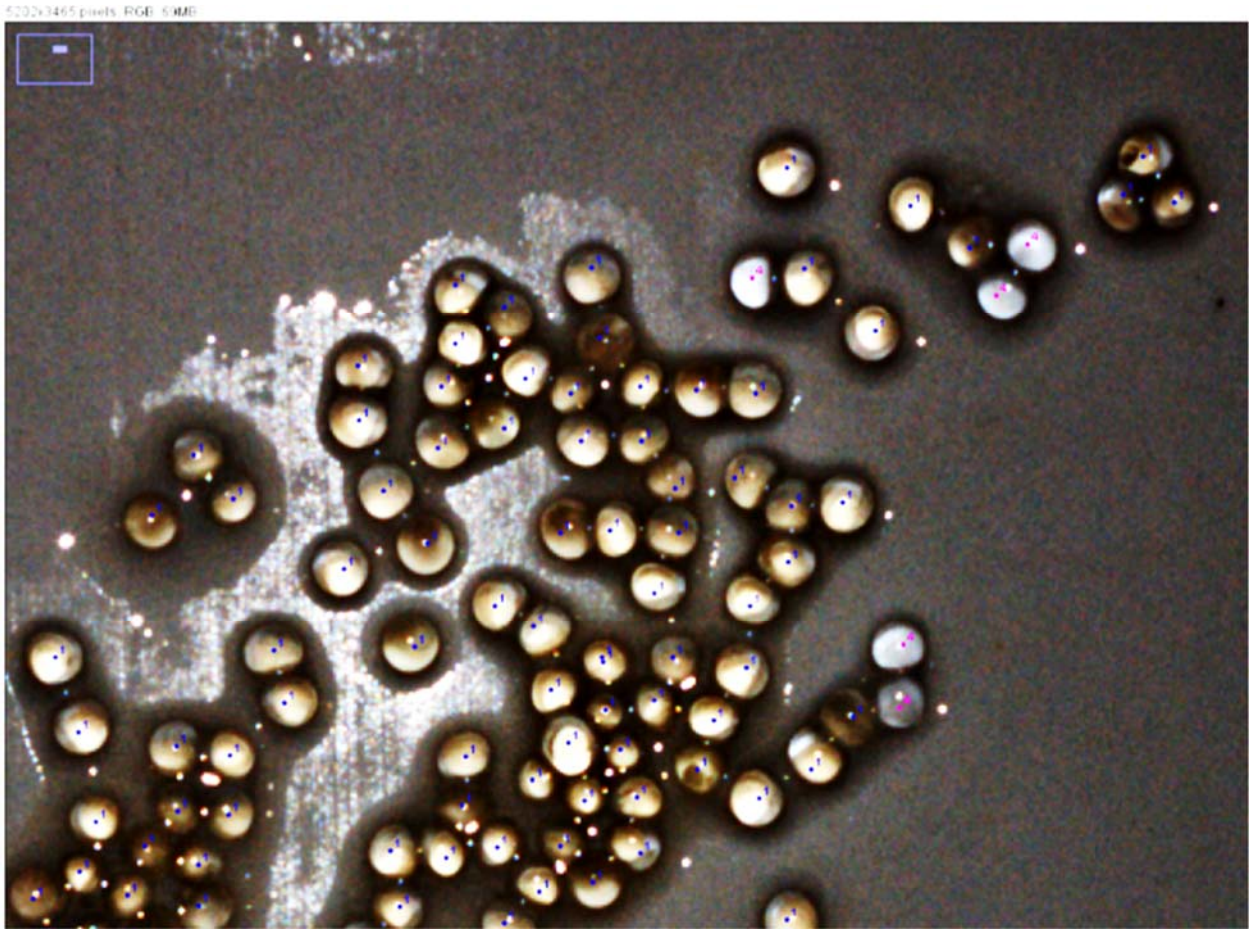

Figure 4-3. Contrast-adjusted photograph of a breeding substrate produced with ImageJ. The Cell Counter plugin has been used to mark and tally fertilized and unfertilized eggs. In this example, fertilized eggs are marked with a blue 1, and unfertilized eggs are marked with a pink 4. Fertilization state based on contrast-adjusted photographs was verified by random follow up sampling after two days.

The testing procedure remained the same for the exposure phase, except for the addition of salinity and video cameras. Because fathead minnows typically spawn in the morning (Ankley et al., 2001), behavioral recordings occurred daily from 9:30 to 10:00 (1.5 h after the lights came on). Cameras were randomly assigned to one tank from each treatment, and an extra camera was randomly assigned to another tank from any treatment, as long as that tank had not been previously recorded multiple times. This assignment procedure allowed all tanks to be recorded at least once, and many were recorded twice. The videos were then assigned random identification numbers, any recorded tank numbers were obscured, and behavioral data were generated using JWatcher + Video 1.0 (Blumstein, Evans, & Daniels, 2006). JWatcher is a program which allows the association of specific behaviors in a video with keystrokes. Thus, data for multiple behaviors can be generated concurrently. Additionally, the user can pause, rewind, and slow the video in order to generate very precise data collection. For tanks with multiple recordings, the generated data were averaged to form a composite score in order to avoid pseudoreplication.

Using ImageJ and JWatcher, a single experimenter generated data for numerous egg and behavioral endpoints. Specifically, the egg endpoints included total eggs produced, percent fertilization, number of spawning days, and clutch size (number of eggs deposited on an individual breeding substrate). Though hatching success is another common endpoint in short-term reproductive assays, it was not evaluated here due to fungal growth on several egg clutches in the static incubation tanks. Behavioral endpoints were based on Cole and Smith (1987), and included number of times males approached females, time spent in nest care, average duration of nest care bouts, time (and average duration) spent in the nest by both males and females, number of spawning attempts during the observation periods, and number of nest defense acts.

Daily water quality parameters (conductivity, pH, dissolved oxygen, and temperature) were recorded for two randomly chosen tanks per treatment with a YSI probe (Professional Plus, YSI Inc., Yellow Springs, OH). Additionally, five ml of tank water was withdrawn from each tank and combined by treatment group for analysis by an independent laboratory (Saskatchewan Research Council, Saskatoon, Canada) for verification of ion composition and salinity concentration (sum of ions).

4.3.6 Statistical analyses

Statistical analyses were performed with R version 2.13.2 (R Development Core Team, 2011). All data were checked for conformance to test assumptions (normality and homogeneity of variance) using R's built-in diagnostic plots and the Fligner-Killeen test for homogeneity of variances. Data were transformed as noted below when those assumptions were not met. Due to mortalities during the exposure phase, three tanks in the control group and one in the 1000 ppm group were removed from all analyses.

4.3.6.1 Assignment for exposure phase

To ensure that there were no confounding differences among salinity treatments before exposure, a 1-way MANOVA was performed using total eggs, male length, male weight, female length, female weight, and pre-exposure temperature as response variables.

4.3.6.2 Water quality

Water quality parameters (conductivity, pH, dissolved oxygen, and temperature) were compared among salinity levels using a 1-way MANOVA. ANOVAs were then performed to investigate which of the parameters differed significantly among levels. Because of significant differences among treatments for pH and temperature, generalized linear models were created for each significant response variable (see Section 4.4.2 Water quality) to determine if the water quality variables were responsible for the significant differences. Because pH and conductivity were highly correlated ($r = 0.84$), saturated models included the following terms as explanatory variables: conductivity * pH + temperature. Saturated models were simplified using stepwise deletion based on residual deviance and/or AIC.

4.3.6.3 Egg and behavioral endpoints

The following measures did not meet required assumptions, and were therefore transformed: total eggs, percent fertilization, number of spawning days, clutch size, and total time spent in nest care.

Percent fertilization was arcsine transformed, and the other measures were Box-Cox power transformed (Table 4-2).

One-way ANOVAs were used to determine the effect of salinity treatment on individual egg and behavioral endpoints. Some measures, such as percent fertilization required the removal of pairs that did not spawn during the entire exposure phase. Additionally, a number of behavioral observations were removed (Control [number removed = 4], 1000 ppm [5], 4000 ppm [4], 8000 ppm [4]) as lab personnel inadvertently entered the room during recording.

Table 4-2. Lambda values for Box-Cox transformations of reproductive endpoints.

Endpoint	Box-Cox lambda
Total eggs produced	0.25
Number of spawning days	0.426
Clutch size	0.25
Total time spent in nest care	0.333

4.4 Results

4.4.1 Assignment for exposure phase

Standard length (mean ± SD: males 5.8 ± 0.5 cm, females 4.6 ± 0.4 cm), weight (males 4.5 ± 0.7 g, females 2.1 ± 0.4 g), pre-exposure egg production (624 ± 278 eggs), and pre-exposure temperature (27.6 ± 1.0°C) did not differ among experimental groups (Pillai's Trace, $F_{(3, 53)} = 1.3$, $p = 0.2$). Size-matching pairs resulted in females averaging 78 ± 3% the length and 48 ± 9% the weight of males.

4.4.2 Water quality

A significant difference in water quality parameters was found among treatment groups (Pillai's Trace, $F_{(3, 53)} = 26$, $p < 0.001$, Table 4-3). No difference was found among treatment groups for dissolved oxygen ($F_{(3, 53)} = 1.7$, $p = 0.17$). However, as predicted, conductivity ($F_{(3,53)} = 2035$, $p < 0.001$), and pH ($F_{(3,53)} = 114$, $p < 0.001$) varied among groups. These differences were predicted because conductivity

increases with increasing salinity and pH tends to increase at the same time. Saline waters (salinity >3000 ppm, Williams, 1964) tend to have higher pH values than freshwaters because 1) the salts in saline waters are involved in acid–base interactions in the water, and 2) increased alkalinity (due to increased concentrations of HCO^{-}_{3} and $CO^{2}_{3}{}^{-}$) raises the equilibrium pH of saline water (Hinga, 2002; Morel & Hering, 1993). Although our tanks were randomized throughout the room, we found a significant difference in temperature among the groups ($F_{(3,53)} = 11$, $p < 0.001$), although the effect was very small (see Table 4-3). Using stepwise deletion of saturated general linear models, conductivity was found to be the only significant explanatory water quality parameter for total eggs ($F_{(1, 55)} = 240$, $p < 0.001$), percent fertilization ($F_{(1, 40)} = 23$, $p < 0.001$), number of spawning days ($F_{(1, 55)} = 28$, $p < 0.001$), clutch size ($F_{(1, 42)} = 99$, $p < 0.001$), total time in nest care ($F_{(1, 38)} = 15$, $p < 0.001$), and average duration of nest care bouts ($F_{(1, 38)} = 17$, $p < 0.001$).

Table 4-3. Mean (± SD) water quality parameters. Percent error for sum of ions is based on the actual sums compared to nominal theoretical values.

Treatment Group	Control	1000	4000	8000
Conductivity (µS/cm)	482 (11)	1650 (79)	4829 (242)	8287 (506)
Dissolved Oxygen (%)	81.5 (4.2)	77.5 (4.8)	79.8 (4.9)	78.5 (5.0)
pH (pH units)	8.0 (0.1)	8.0 (0.1)	8.4 (0.1)	8.4 (0.1)
Temperature (°C)	27.8 (0.3)	29.2 (0.7)	29.2 (1.0)	28.9 (0.8)
Sum of ions (ppm)	303	997	4140	8230
% Error	---	0%	4%	3%

4.4.3 Egg and behavioral endpoints

Salinity affected the average number of eggs produced per pair during the exposure phase ($F_{(3,53)} = 17$, $p < 0.001$, Figure 4-4a), with all salinity-exposed groups producing fewer eggs than control (Tukey comparisons: all $p < 0.05$), and the 4000 ppm and 8000 ppm treatments producing fewer eggs than the 1000 ppm treatment (both $p \leq 0.01$). Percent fertilization was also affected by salinity ($F_{(3,38)} = 11$, $p < 0.001$, Figure 4-4b). However, only the 8000 ppm treatment group was affected; this group showed

significantly reduced percent fertilization when compared to all other treatments (all $p \leq 0.002$). Number of spawning days was also affected by salinity ($F_{(3,53)} = 11$, $p < 0.001$, Figure 4-4c). There was no difference between control and 1000 ppm ($p = 0.38$) and no difference between 4000 ppm and 8000 ppm ($p = 0.99$), but the 4000 ppm and 8000 ppm groups spawned on fewer days than the control and 1000 ppm groups (both $p \leq 0.01$). The final egg endpoint, average clutch size, was also affected by salinity ($F_{(3,40)} = 5.5$, $p = 0.003$, Figure 4-4d), with no difference between control and 1000 ppm ($p = 0.71$). A smaller average clutch size was produced in the 4000 ppm and 8000 ppm groups than in control (both $p \leq 0.02$), but interestingly, there was no difference among 1000 ppm, 4000 ppm, and 8000 ppm (all $p > 0.05$).

Of the nine behavioral endpoints measured, only two, total time spent in nest care and average duration of nest care events, were significantly affected by salinity. For the total time spent in nest care endpoint ($F_{(3,36)} = 3.9$, $p = 0.02$, Figure 4-5a), only the 8000 ppm group spent significantly less time caring than control fish (Tukey comparison $p = 0.02$). However, the 1000 ppm, 4000 ppm, and 8000 ppm groups were not significantly different (all $p > 0.05$). For average duration of nest care events ($F_{(3,36)} = 5.8$, $p = 0.002$, Figure 4-5b), the 8000 ppm group had significantly shorter bouts than the control and 1000 ppm groups (both $p \leq 0.01$), however, there was no significant difference between 4000 ppm and 8000 ppm ($p = 0.6$) or control and 1000 ppm ($p = 0.99$).

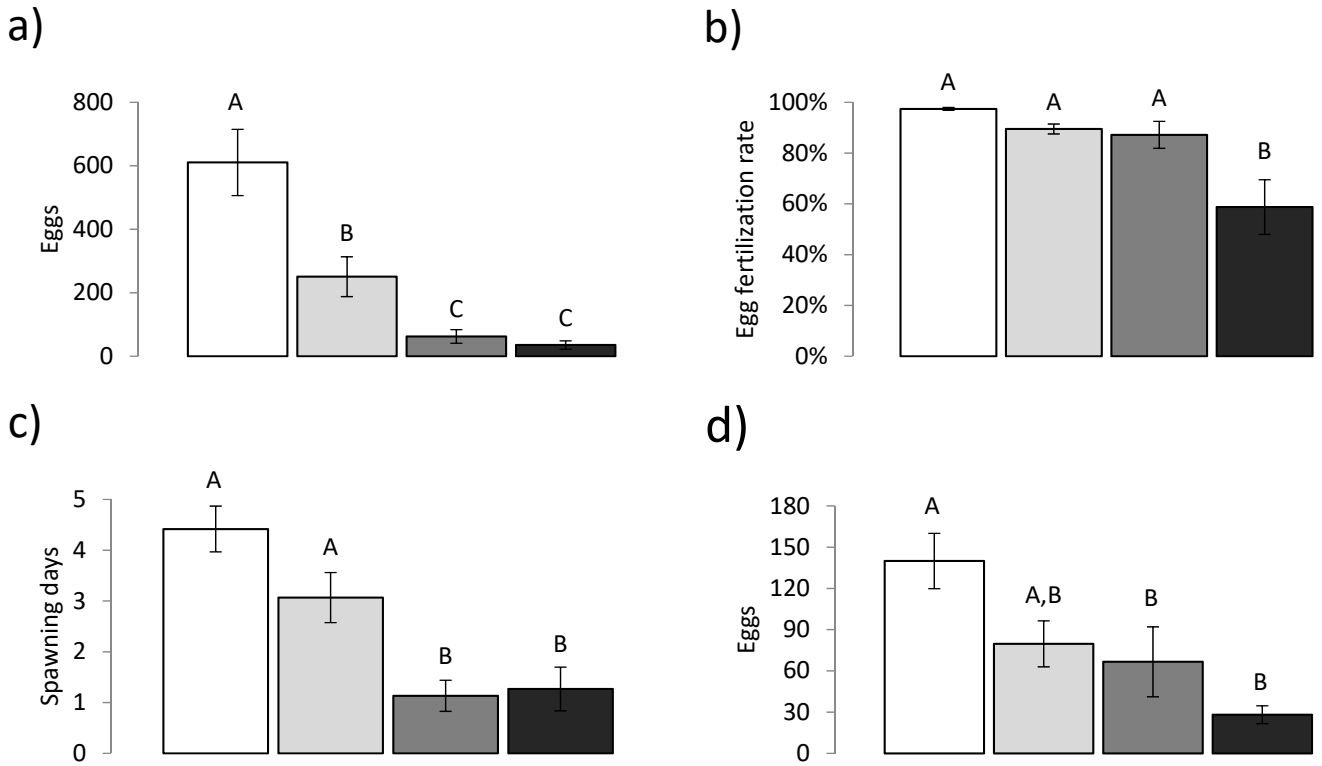

Figure 4-4. Mean (±SE) egg based endpoints. Minnows (n = 15 per treatment) were exposed to dechlorinated tap water (Control, white bars), 1000 ppm (light gray bars), 4000 ppm (dark gray bars), or 8000 ppm (black bars) over a 21-day exposure phase. Data recorded includes: a) total eggs produced per pair, b) percent fertilization, c) number of days when spawning took place, and d) clutch size. Different capital letters above the error bars indicate significant differences at alpha of 0.05.

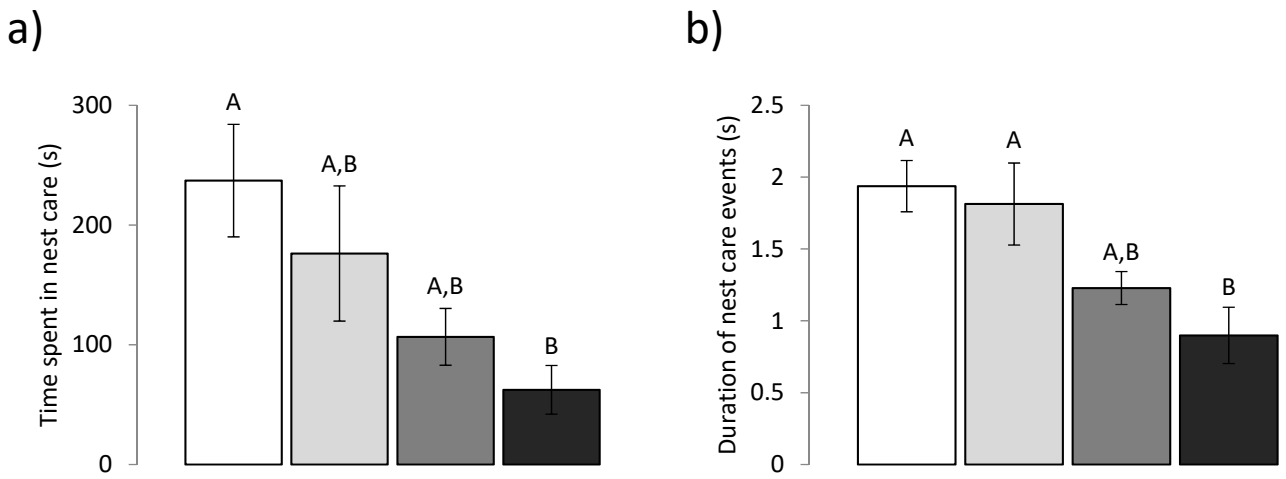

Figure 4-5. Mean (± SE) behavioural endpoints. Minnows (n = 15 per treatment) were exposed to dechlorinated tap water (Control, white bars), 1,000 ppm (light grey bars), 4,000 ppm (dark grey bars), or 8,000 ppm (black bars) over a 21-d exposure phase. Data recorded includes: a) time spent in nest care by males, and b) duration of nest care events. Different capital letters above the error bars indicate significant differences at alpha of 0.05.

4.5 Discussion

Our study demonstrates that sub-lethal concentrations of salinity can adversely impact a number of reproductive endpoints, including: total egg production, percent fertilization, number of spawning days, clutch size, time spent in nest care, and duration of nest care events. These effects were generally more pronounced in the two highest salinities, 4000 ppm and 8000 ppm. Of the egg based endpoints, total eggs produced seemed to be the most sensitive, and percent fertilization the least. This distinction seems to suggest that egg production endpoints are affected most strongly by either increased metabolic costs or a disruption of chemical cues, while fertilization success is likely influenced most strongly by the specific concentration of ions in the water. Indeed, Rosengrave et al. (2009) found that the sperm longevity of chinook salmon (*Oncorhynchus tshawytscha*) was negatively correlated with Ca^{2+} and Mg^{2+} concentrations in the ovarian fluid surrounding eggs. It is likely that fathead minnow sperm longevity is similarly affected by saline water with high concentrations of these ions. Given that the concentration of Ca^{2+} and Mg^{2+} in the 8000 ppm treatment was roughly double that of the 4000 ppm treatment (4.5 and 77.5 mEq/L, 2.9 and 39.4 mEq/L, respectively), this seems a likely explanation for the observed reduction in fertilization success in the highest salinity treatment.

Though the metabolic costs of exposure to salinity were not directly measured, they likely increased with increasing salinity, and played a role in the observed effects on total eggs, number of spawning days, and average clutch size. For instance, as the salinity concentration increased, the minnows likely had to up-regulate the active transport of ions. This would lead to increased resources devoted to osmoregulation, and decreased resources available for reproduction. These metabolic costs were likely accompanied by yet another probable mechanism— the disruption of chemical cues. It is well established that many fishes excrete a number of chemical cues in order to coordinate reproductive functions (Stacey & Sorensen, 2005). Additionally, recent research has shown that territorial male fathead minnows (i.e. those which have secured a breeding territory) excrete cues in their urine to convey social status, and therefore, fitness as a mate (Martinovic-Weigelt et al., 2012). Should stenohaline freshwater fish in a saline environment not be able to maintain osmotic regulation, they may become dehydrated, and quickly lose the ability to excrete chemical cues. This would most likely lead to poor communication of reproductive state to potential mates, and may help explain the observed effects.

At the outset of this study, we predicted a link between reproductive behavior and egg production endpoints. Though we measured nine behavioral endpoints, only two, average time spent in

nest care and average duration of nest care bouts, showed significant effects. We believe that the lack of effect in other behaviors is at least partially attributable to the accidental presence of personnel in the laboratory during some of the behavioral recordings. Upon review of the recordings, this accidental presence clearly affected the fish, as they generally stopped any reproductive behaviors they were undertaking and switched to monitoring behaviors. This led to a number of observations being thrown out, which reduced samples sizes, and likely increased within-group variance. The two significant behavioral endpoints, time spent in nest care and average duration of nest care events, were likely more robust than some of the other endpoints. This is because males spend time in nest care when in reproductive state, regardless of the presence of eggs or receptivity of females. Despite the interruption in behavior recording, the significant effects of salinity on nest care are important because a male which does not adequately perform these stereotypic behaviors may not be able to attract a female or properly care for any eggs that are deposited and fertilized.

Increasing salinity poses a major threat to many inland water bodies, particularly when secondary salinization compounds primary salinization. Though much research has been undertaken on the lethal impacts of salinization, it is important to consider the effects of sub-lethal salinity increases. After all, though a fathead minnow can survive a substantial salinity increase, the impairment of vital activities such as reproduction could be just as devastating to the population as outright toxicity. Future experiments aimed at determining the population-level effects of salinity-induced reductions in breeding are in order. In many small lakes and ponds across the Great Plains a reduction in density of fathead minnows is associated with dramatic alterations in nutrient dynamics, water quality, and other ecosystem characteristics in these prairie wetlands (Zimmer et al., 2002). With an increase in both primary and secondary salinization predicted to occur in much of the prairies, we need to consider the potential for long-term acclimation to increased salinity, and whether fathead minnows and other species might be able to eventually overcome the challenges of salinization. Although it will be challenging, we also need to begin to examine any generational effects of exposure to salinization.

Chapter 5: Study 4—Impact of salinity change on acclimated fathead minnow (*Pimephales promelas*) reproduction

This study contains unpublished data.

5.1 Abstract

Salinization can impact inland aquatic ecosystems in numerous ways. It is important to note that increased salinity can have negative effects, not only on survival, but also on important activities and behaviours. These important activities can be impacted at relatively low salinity levels. Reproduction in fathead minnows (*Pimephales promelas*) is one such important activity affected by increases in salinity. In order to determine whether these negative impacts could be overcome through acclimation to low-level salinity, and whether this acclimation point, should it exist, would impact minnows' responses to subsequent changes in salinity, I undertook several short-term reproductive assays. Minnows were exposed to 1000 ppm salinity for various periods (2, 6, 10, or 14 weeks), and their reproductive output was measured using four egg-based endpoints. After the 14-week exposure, I found that total eggs produced per pair, percent fertilization, number of spawning days, and average clutch size did not differ between fish exposed to salinity and control fish only exposed to dechlorinated tap water. I have, therefore, potentially determined an adequate acclimation point for reproductive endpoints of fathead minnows exposed to 1000 ppm salinity. It must be noted, however, that my scope of inference may be limited by my small sample size in the 14-week exposure trial.

5.2 Introduction

Primary, or non-anthropogenic, salinization and secondary (anthropogenic) salinization are concerns for many inland water bodies, and can be particularly damaging when they work together. Aquatic communities tend to be fairly resistant to small salinity changes over time, but may not be able to cope if those changes are dramatic or rapid (Flower, 2001). When changes are dramatic or rapid, increased salinity has been shown to cause shifts in biotic communities, limit biodiversity, exclude less tolerant species, and cause acute or chronic effects at specific life stages (Weber-Scannell & Duffy, 2007). In fact, if left unchecked, salinization could render many inland water bodies unfit for animal and/or human use (Williams, 1987).

Numerous studies have examined the physiological effects of salinization on aquatic organisms. However, the vast majority focus on NaCl alone (for example, Bezirci et al., 2012; Pistole et al., 2008). While Na^+ and Cl^- are the major ions found in many salinized water bodies around the world and are therefore important to study, other regions can be dominated by different ions (such as the $MgSO_4$ and $NaSO_4$ dominated lakes of the Canadian Prairies). This is an important distinction because the ion ratio of

water has been shown to have dramatic physiological effects. For instance, Mount et al. (1997) found the 96-h LC50 for fathead minnows (*Pimephales promelas*) ranged from <510 to 7960 parts per million (ppm) based on the ion ratio and salts present in the experimental water. Similarly, for rainbow trout (*Oncorhynchus mykiss*) and larval chironomids (*Chironomus tentans*), Chapman et al. (2000) found that the toxicity of mining effluents was not predictable based on total dissolved solids (TDS) concentration alone, but instead depended upon the combination and concentration of ions in the water.

In addition to overcoming the physiological stress imposed by salinity, aquatic animals must also successfully forage, mate, and avoid predators. In previous work, we have shown that some of these important activities can be negatively impacted by increases in salinity which are well below physiological tolerance levels. We have previously shown that sub-lethal salinity concentrations can reduce both antipredator behaviour (Hoover, Ferrari, et al., 2013) and reproduction, both behaviour and egg-based endpoints (Hoover, Weisgerber, et al., 2013), in fathead minnows.

Given that a relatively rapid exposure to a salinity concentration as low as 1000 ppm has been shown to negatively impact fathead minnow reproduction (Hoover, Weisgerber, et al., 2013), that aquatic communities tend to be resilient to small salinity changes over time (Flower, 2001), and that fathead minnows are found in inland water bodies with salinity concentrations up to approximately 10,000 ppm (Rawson & Moore, 1944), I was interested to investigate whether fathead minnows could reach an acclimation point wherein fecundity measures of minnows held in 1000 ppm salinity would match those of minnows held in dechlorinated tap water (≈ 300 ppm). Additionally, should an acclimation point be found, I was interested in examining the effects of changing salinity on fully-acclimated minnows. To pursue these ends, I performed several pilot studies using short-term reproductive assays (OECD, 2009; USEPA, 2002). I predicted minnows would reach an acclimation point, and that this acclimation might impact the minnows' response to subsequent changes in salinity.

5.3 Methods

5.3.1 Experimental Design

Using a completely randomized and fully blind short-term reproductive assay, I planned to test the effects of salinity change on several fathead minnow reproductive endpoints, once minnows had been acclimated to 1000 ppm salinity (n = 8 pairs per treatment). This experiment consisted of several

rounds of pilot studies (acclimation periods of 2, 6, 10, and 14 weeks; each pilot study lasting 3 weeks). The pilot studies were designed to help determine an adequate acclimation period wherein fecundity measures of acclimated fish would match those of non-acclimated fish held in dechlorinated tap water (control). After an acclimation period was identified (or after six months, should no acclimation occur) I planned to expose fish held in 1000 ppm to 250 ppm, 1000 ppm (control), 4000 ppm, and 8000 ppm.

5.3.2 Experimental fish

Adult fathead minnows were purchased from a commercial supplier (Osage Catfisheries Inc., Osage Beach, MO, USA) and housed according to sex in four 40 L recirculating tanks filled with dechlorinated tap water (salinity ≈ 300 ppm). The minnows were fed commercial fish flakes (Nutrafin Max Flake Food, Rolf C. Hagen Inc., Montreal, QC, Canada) *ad libitum* at 9:00 and 16:00 daily. Additionally, the 16:00 feeding was supplemented with freeze dried blood worms (Omega One, Omega Sea Ltd., Painesville, OH, USA). Minnows were held at approximately 25 °C with a 16:8-hour light:dark photoperiod and at least 75% oxygen saturation. The fish were maintained in these conditions for two weeks prior to the beginning of the experiment in order to ensure their health and acclimation to the laboratory environment.

5.3.3 Salinity preparation

Experimental water was prepared by adding the salts listed in Section 4.3.3 Salinity preparation to dechlorinated tap water. See Figure 5-1 for a representation of the milligram equivalent per liter (mEq/L) percent ion composition of the experimental water for each pilot study.

Experimental water was prepared by adding the salts and dechlorinated tap water to mixing tanks (150 L volume). The solutions were then circulated within the mixing tanks overnight. This approach aided in the dissolution of salts, and also aided in bringing each solution to room temperature. Control water followed the same procedures as saline water. The acclimation period utilized a recirculating system. Therefore, mixing tanks holding experimental water were replenished daily with dechlorinated tap water in order to counteract losses due to evaporation. Additionally, all water was flushed from the system and new water was prepared every two weeks.

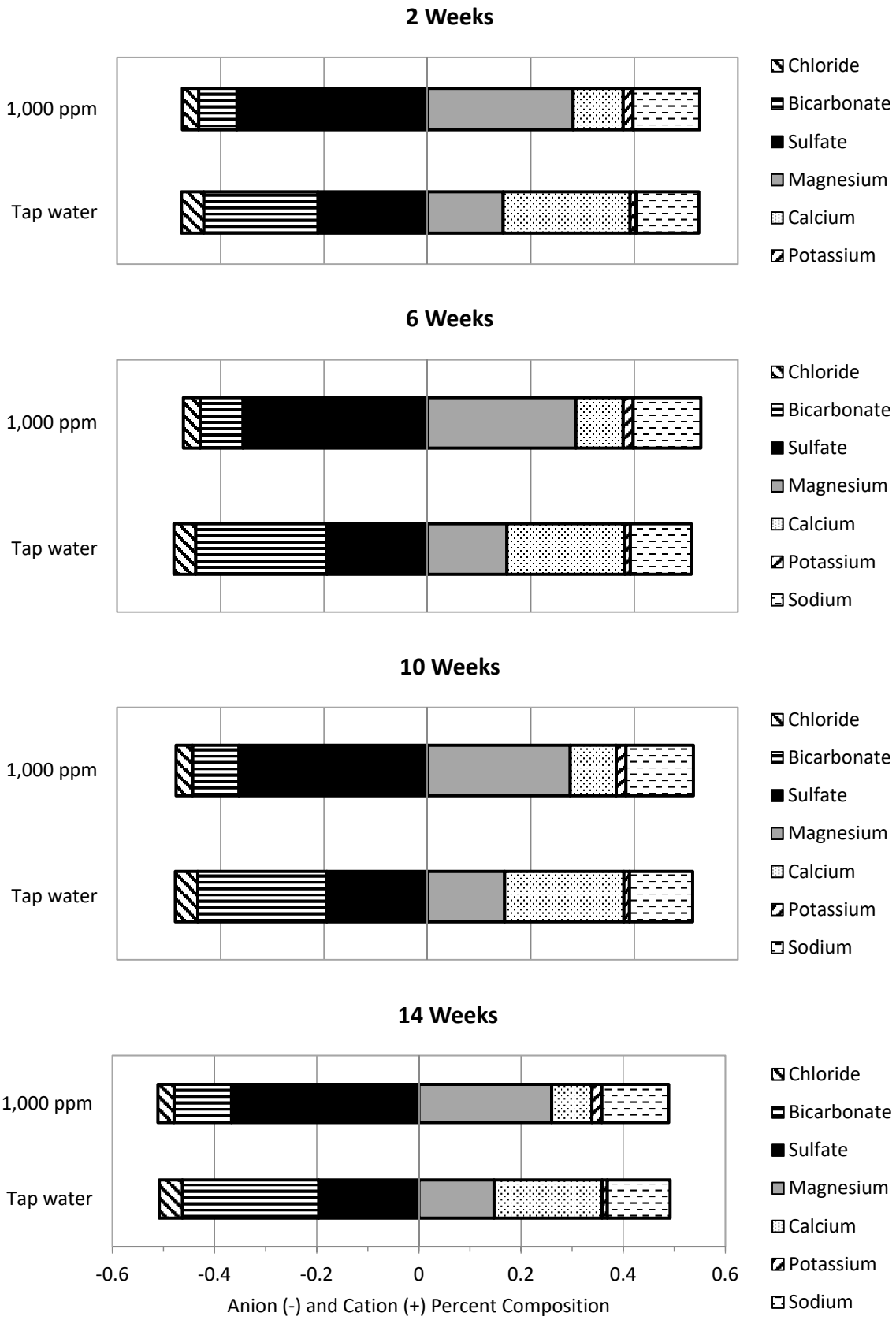

Figure 5-1. Average milligram equivalent per liter ion composition for control and experimental waters for each of the four pilot studies. The ion composition of experimental waters is based on sampling of Lake Lenore by the Saskatchewan Watershed Authority (2007–2008). Values are based on the results of independent laboratory analysis (Saskatchewan Research Council, Saskatoon, Canada). Ions are divided into anions and cations. Note that percent composition should be considered an absolute value; anions are represented as negative numbers to facilitate comparison.

5.3.4 Test apparatus

The test apparatus was a recirculating system consisting of mixing tanks, head tanks for distribution, and acclimation and spawning tanks which drained into Laguna PowerFlo 1000 External Biological Filters (Rolf C. Hagen Inc., Montreal, QC, Canada). The filters returned water to the mixing tanks (See Figure 5-2). There were two systems—one circulating dechlorinated tap water, and one circulating 1000 ppm water. Each system contained two types of fish-holding tanks, acclimation tanks and spawning tanks. Acclimation tanks were the same tanks used to acclimate fish to laboratory conditions (Section 5.3.1 Experimental Design), 40 L glass aquaria with drains, gravel substrates, and air stones. Fish were housed in the acclimation tanks until testing at designated time periods (2, 6, 10, and 14 weeks). For testing, fish were randomly assigned to spawning tanks (N = 32, 8 pairs per treatment) containing water of the same salinity as their acclimation tanks. Spawning tanks consisted of nine L plastic aquaria with drains, lids, air stones, and breeding substrates (10 cm diameter PVC pipe cut in half, 7.5 cm long, one per aquarium). All tanks, acclimation and spawning, were blacked out on three sides to prevent visual cues from adjacent tanks. Additionally, filters were inspected weekly and cleaned if necessary.

One size-matched pair was placed in each spawning tank (females ≈ 76% the length of males, averaged across all four pilot studies). A size-matched approach was chosen because, when compared to randomly assigned trios of minnows, this approach has been shown to both reduce the number of animals used and increase breeding efficacy (Pollock et al., 2008). Pairs remained in the spawning tanks for three weeks. During this time, the pairs were only disturbed for feeding, to check breeding substrates, collect water samples, and to perform any necessary tank cleaning. After spawning trials, fish were moved to separate holding tanks according to salinity, and were not used again.

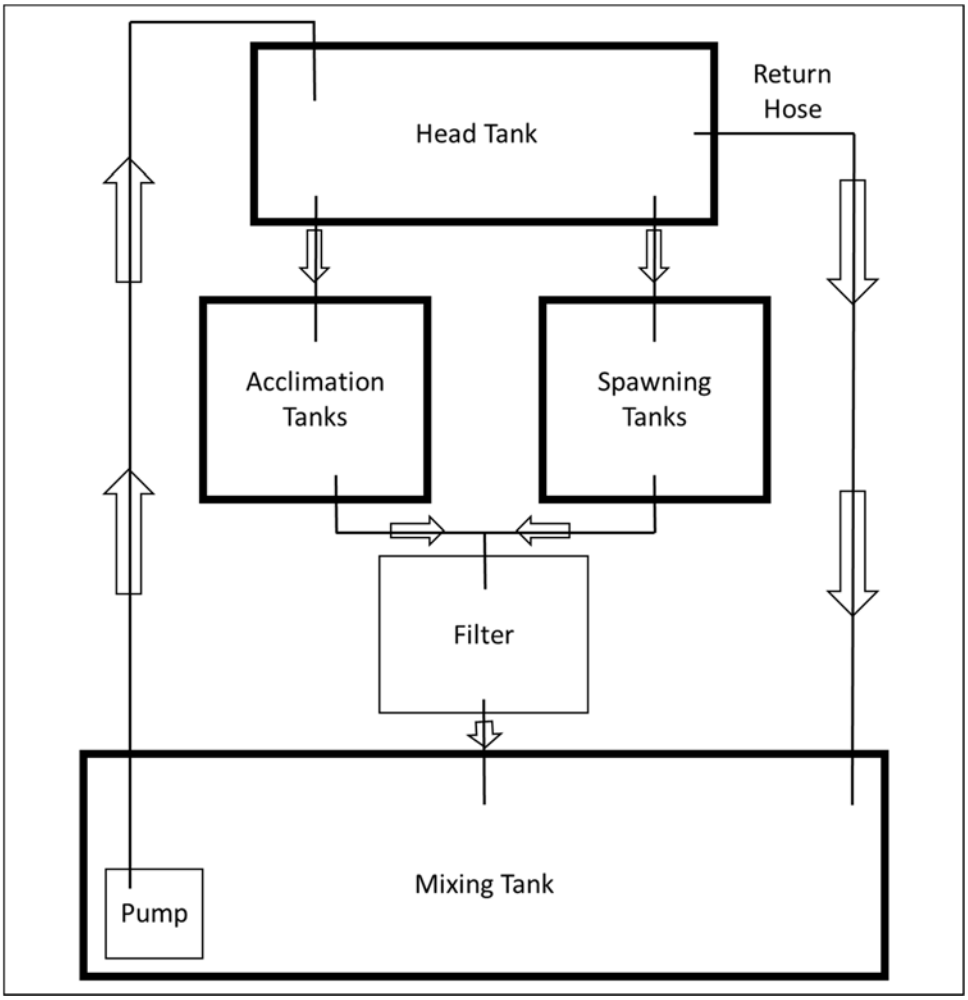

Figure 5-2. Test apparatus setup. Experimental water was continually circulated through the system. Water was first prepared in the mixing tanks, then pumped to the head tanks at a rate greater than required for distribution to the acclimation/spawning tanks. This allowed water to return to the mixing tanks through the return hose, ensuring a consistent head pressure for distribution. Water from the head tanks was distributed to the acclimation/spawning tanks, where it then overflowed, and proceeded to the filters. Water from the filters then returned to the mixing tanks.

5.3.5 Testing procedure and data collection

Breeding substrates were checked daily following the protocol outlined in Section 4.3.5 Testing procedure and data collection. After being checked, substrates were returned to their holding tanks, and the eggs were allowed to hatch. Larvae were reared to adulthood, and used in a subsequent study (Study 5—Impact of salinity on second-generation fathead minnows (*Pimephales promelas*)). Using ImageJ, a single experimenter generated data for the following egg-based endpoints: average total eggs produced per pair, percent fertilization, number of spawning days over the three-week trial, and clutch size.

Daily water quality parameters (conductivity, pH, dissolved oxygen, and temperature) were recorded for all spawning tanks with a YSI probe (Professional Plus, YSI Inc., Yellow Springs, OH). Additionally, five ml of tank water was withdrawn from each tank at the end of each pilot study and combined by treatment group for analysis by an independent laboratory (Saskatchewan Research Council, Saskatoon, Canada) for verification of ion composition and salinity concentration (sum of ions).

5.3.6 Statistical analyses

Statistical analyses were performed with R version 3.2.3 (R Development Core Team, 2015), using the RStudio IDE, version 0.99.491 (RStudio Team, 2015). All data were checked for conformance to test assumptions (normality and homogeneity of variance) using R's built-in diagnostic plots and the Fligner-Killeen test for homogeneity of variance. Data were transformed as noted below when those assumptions were not met.

5.3.6.1 Assignment to spawning tanks

To ensure there were no differences between treatment groups for physical characteristics before spawning trials began, a 1-way MANOVA was performed for each pilot study using male length, male weight, female length, and female weight as response variables.

5.3.6.2 Water quality

Water quality parameters (conductivity, pH, dissolved oxygen, and temperature) were compared between salinity treatments using a 1-way MANOVA for each pilot study. Individual ANOVAs were then performed to investigate which of the parameters differed significantly between levels.

5.4.6.3 Egg-based endpoints

The egg-based measurements did not meet the required assumptions, and were therefore transformed. Percent fertilization was arcsine transformed, while total eggs produced per pair, number of spawning days, and clutch size were Box-Cox power transformed (Table 5-1). Several minnow pairs were removed from egg-based endpoint analyses because they did not produce any eggs during the trial (2-week acclimation pilot study: one pair from the 1000 ppm treatment; 14-week acclimation pilot study: four pairs from the 1000 ppm treatment, one pair from the dechlorinated tap water treatment).

Table 5-1. Lambda values for Box-Cox transformations of reproductive endpoints for pilot study 1 (6-week acclimation period) and pilot study 4 (14-week acclimation period).

Endpoint	Box-Cox lambda (pilot study 1)	Box-Cox lambda (pilot study 4)
Total eggs produced	1.68	0.446
Number of spawning days	1.1	0.655
Clutch size	0.532	0.989

5.4 Results

In each of the following subsections, pilot studies will be denoted as follows: 2-week acclimation period = ps1 (pilot study 1), 6-week acclimation period = ps2, 10-week acclimation period = ps3, and 14-week acclimation period = ps4.

5.4.1 Assignment to spawning tanks

Standard length and weight did not significantly differ between salinity treatments for any of the four pilot studies (ps1: Pillai's Trace, $F_{(1,14)} = 3.1$, $p = 0.06$; ps2: $F_{(1,14)} = 5.2$, $p = 0.13$; ps3: $F_{(1,14)} = 3.2$, $p = 0.05$; ps4: $F_{(1,14)} = 1.3$, $p = 0.33$). See Table 5-2 for a summary of physical characteristics for each pilot study.

Table 5-2. Mean (± SD) physical characteristics for each pilot study.

Pilot Study	ps1	ps2	ps3	ps4
Male length (cm)	4.4 (0.4)	4.8 (0.3)	4.7 (0.3)	4.9 (0.4)
Male weight (g)	2.1 (0.6)	2.5 (0.3)	2.4 (0.5)	2.4 (0.9)
Female length (cm)	3.4 (0.3)	3.6 (0.2)	3.6 (0.2)	3.7 (0.3)
Female weight (g)	1.2 (0.3)	1.2 (0.3)	1.3 (0.3)	1.5 (0.3)

5.4.2 Water quality

A significant difference in water quality parameters was found between treatment groups for all pilot studies (ps1: Pillai's Trace, $F_{(1,14)} = 550$, $p < 0.001$; ps2: $F_{(1,14)} = 818$, $p < 0.001$; ps3: $F_{(1,14)} = 2379$, $p < 0.001$; ps4: $F_{(1,14)} = 1049$, $p < 0.001$, Table 5-3). As predicted, only conductivity varied between treatment groups. No difference was found between treatments for dissolved oxygen, pH, or temperature. See Table 5-3 for *F* and *p* values.

Table 5-3. Mean (± SD) water quality parameters for each pilot study, including *F* and *p* values for individual ANOVAs. * indicates significant differences at alpha of 0.05. Percent error for sum of ions is based on the actual sums compared to nominal theoretical concentrations.

Pilot Study	ps1			ps2			ps3			ps4		
Treatment	Tap water	1000	$F_{(1,14)}$, *p* value	Tap water	1000	$F_{(1,14)}$, *p* value	Tap water	1000	$F_{(1,14)}$, *p* value	Tap water	1000	$F_{(1,14)}$, *p* value
Conductivity (µS/cm)	532 (35)	1566 (42)	2474, <0.001*	552 (31)	1582 (36)	3277, <0.001*	528 (23)	1591 (19)	8882, <0.001*	554 (31)	1598 (25)	4834, <0.001*
Dissolved Oxygen (%)	83.4 (6.3)	83.5 (5.7)	0.05, 0.97	86.3 (9.9)	86.9 (7.5)	0.02, 0.89	82.3 (8.8)	85.2 (7.3)	0.43, 0.52	82.7 (8.9)	85.7 (3.9)	0.67, 0.89
pH (pH units)	7.90 (0.07)	7.92 (0.06)	0.36, 0.56	7.89 (0.07)	7.93 (0.09)	0.65, 0.43	7.90 (0.09)	7.95 (0.06)	2.2, 0.16	7.9 (0.05)	7.89 (0.05)	0.02, 0.89
Temperature (°C)	26.5 (0.3)	26.8 (0.4)	2.6, 0.13	26.5 (0.4)	26.8 (0.4)	1.9, 0.19	26.4 (0.2)	26.7 (0.6)	1.3, 0.28	26.5 (0.3)	26.8 (0.3)	2.2, 0.15
Sum of ions (ppm)	414	1136	---	384	1078	---	395	1111	---	388	1123	---
% Error	---	14%	---	---	8%	---	---	11%	---	---	12%	---

5.4.3 Egg-based endpoints

5.4.3.1 ps1—2-week exposure to salinity

Fish exposed to 1000 ppm salinity for two weeks produced fewer total eggs per pair than those held in dechlorinated tap water (control) over the three-week spawning trial (ANOVA, $F_{(1,13)} = 6.3$, $p = 0.03$, Figure 5-3a). The percentage of eggs successfully fertilized over the trial period did not significantly differ between groups ($F_{(1,13)} = 0.70$, $p = 0.42$, Figure 5-3b). Similarly, the number of spawning days was not different between groups ($F_{(1,13)} = 0.64$, $p = 0.44$, Figure 5-3c). However, the average clutch size of the control group was significantly larger than the 1000 ppm group ($F_{(1,13)} = 6.4$, $p = 0.03$, Figure 5-3d). See Figure 5-4 for a summary of cumulative eggs produced during this pilot study for each treatment.

5.4.3.2 ps2—6-week exposure to salinity

After day 11 of ps2, no pairs in the control group produced eggs. Therefore, no statistical analyses were performed for this pilot study. See Figure 5-5 for a summary of cumulative eggs produced during this pilot study for each treatment.

5.4.3.3 ps3—10-week exposure to salinity

No pairs produced eggs in either treatment group over the 3-week trial. Therefore, no statistical analyses were possible.

5.4.3.4 ps4—14-week exposure to salinity

Fish did not differ between treatment groups for any of the egg-based endpoints in ps4. No difference was found for total eggs produced per pair (ANOVA, $F_{(1,9)} = 4.0$, $p = 0.08$, Figure 5-6a), percent fertilization ($F_{(1,9)} = 0.1$, $p = 0.76$, Figure 5-6b), number of spawning days ($F_{(1,9)} = 2.1$, $p = 0.18$, Figure 5-6c), or clutch size ($F_{(1,9)} = 1.6$, $p = 0.23$, Figure 5-6d). However, it should be noted that four pairs in the 1000 ppm treatment and one pair in the control group did not produce any eggs during the trial, and therefore were not included in analyses. See Figure 5-7 for a summary of cumulative eggs produced during this pilot study for each treatment.

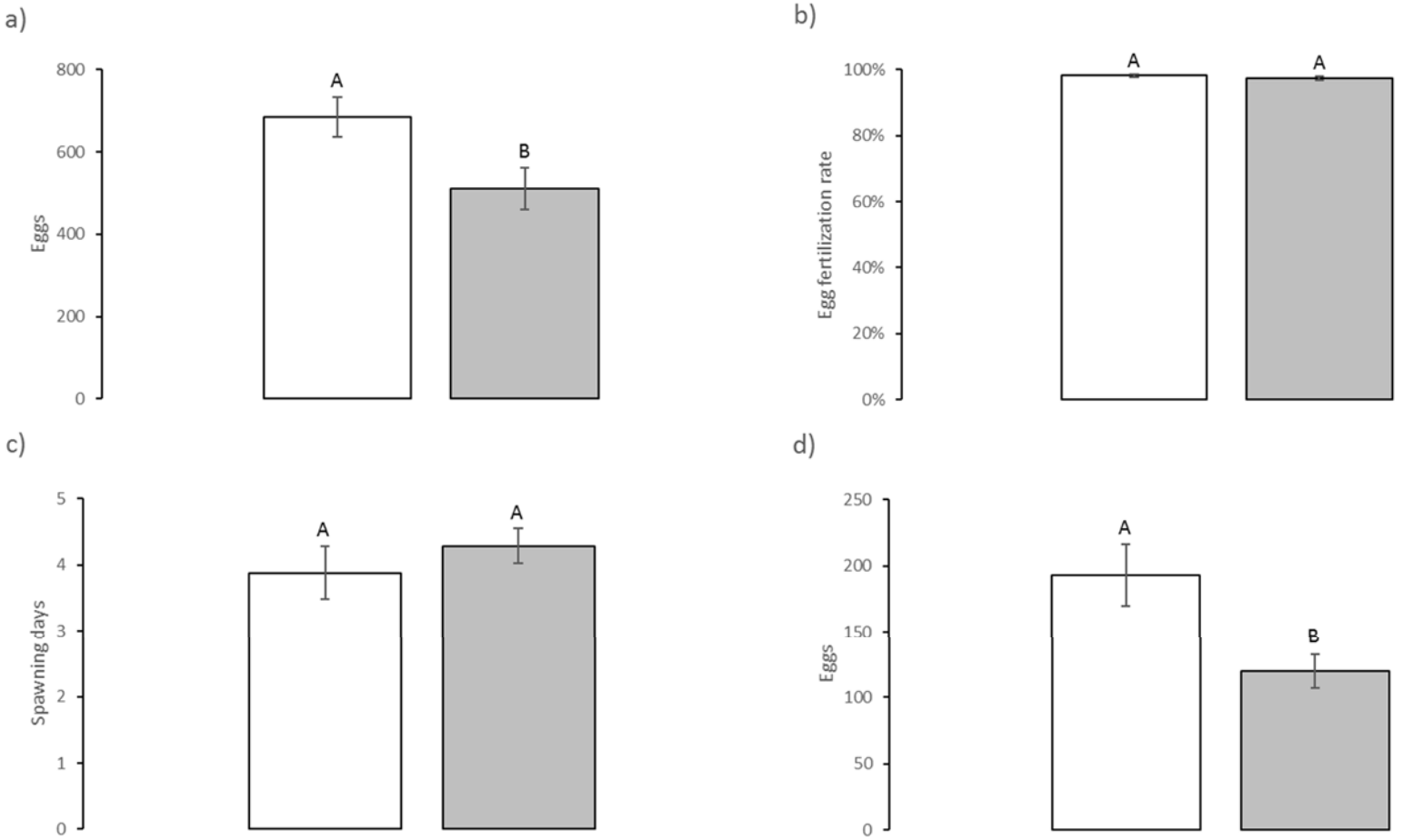

Figure 5-3. Mean (±SE) egg-based endpoints for ps1. Minnows (n = 7 pairs for the tap water treatment, 8 pairs for the 1000 ppm treatment) were acclimated for a 2-week period to tap water (white bars) or 1000 ppm salinity (gray bars), and then tested in corresponding salinity for a 3-week trial. Data recorded includes: a) total eggs produced per pair, b) percent fertilization, c) number of days when spawning took place, and d) clutch size. Different capital letters above the error bars indicate significant differences at alpha of 0.05.

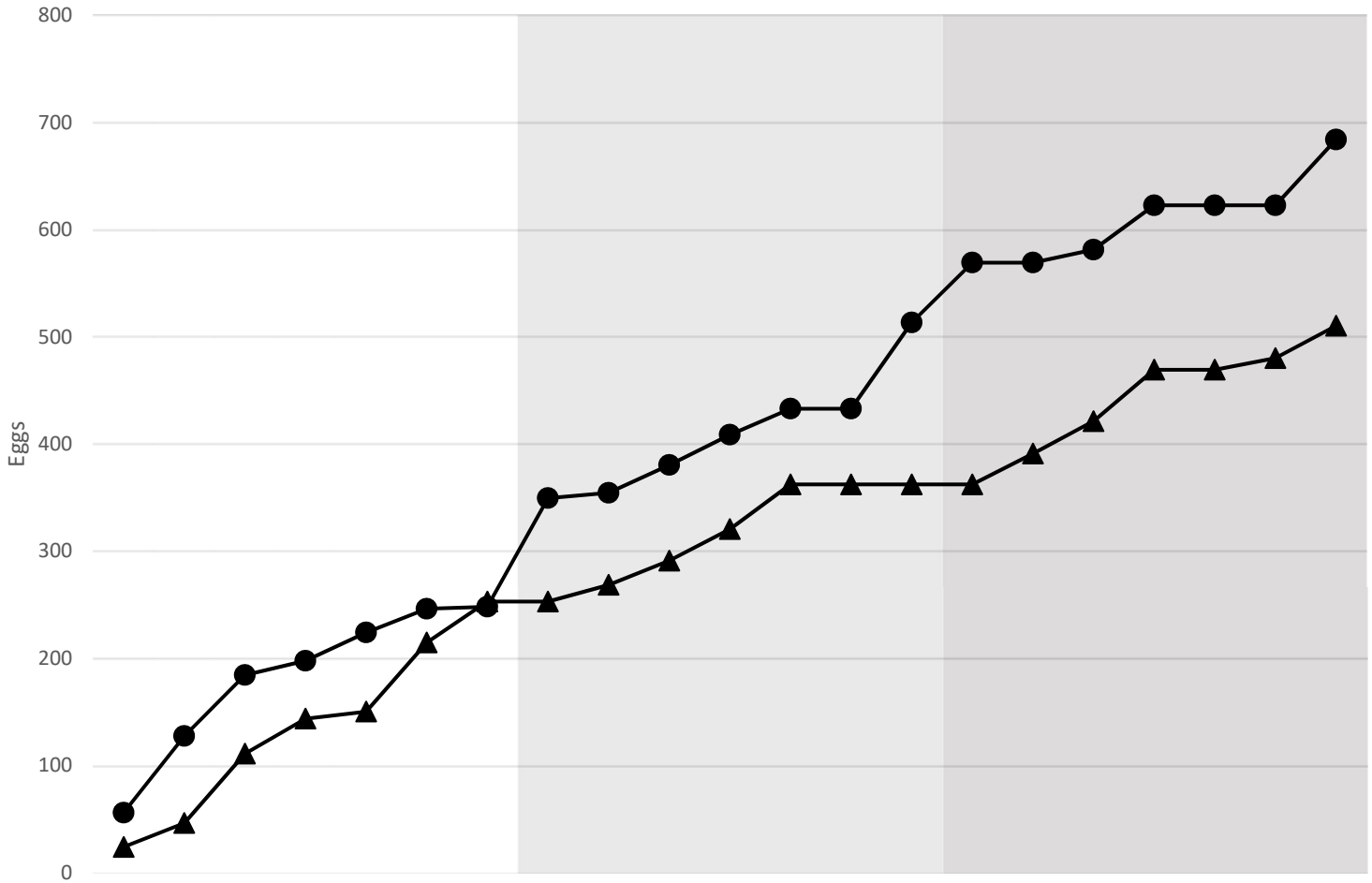

Figure 5-4. Cumulative eggs produced during ps1. Total number of eggs produced for each treatment (tap water represented by circles, n = 7 pairs; 1000 ppm represented by triangles, n = 8 pairs) over the 3-week trial (week 1 = white, week 2 = light gray, week 3 = dark gray).

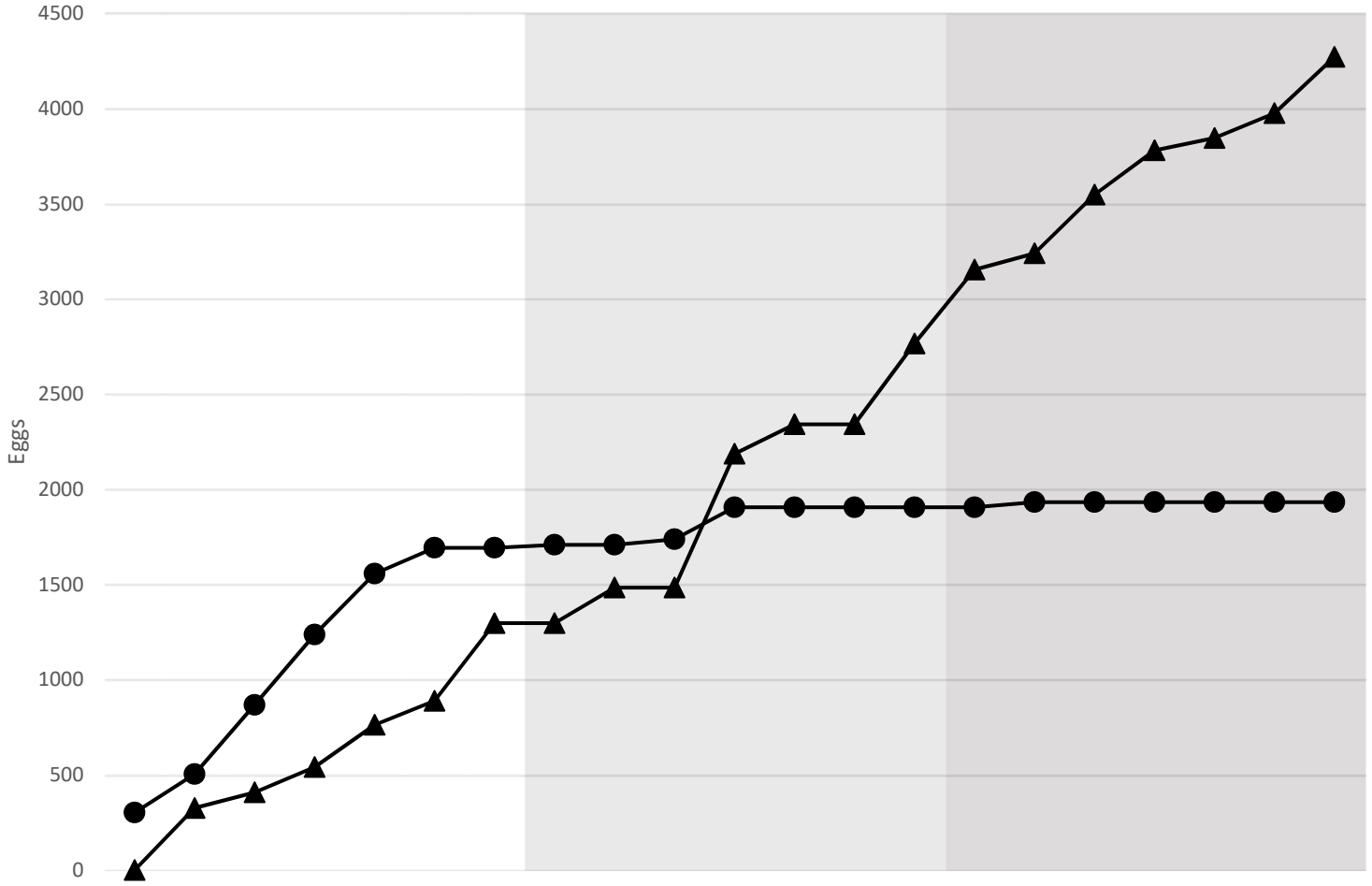

Figure 5-5. Cumulative eggs produced during ps2. Total number of eggs produced for each treatment (tap water represented by circles, n = 8 pairs; 1000 ppm represented by triangles, n = 8 pairs) over the 3-week trial (week 1 = white, week 2 = light gray, week 3 = dark gray).

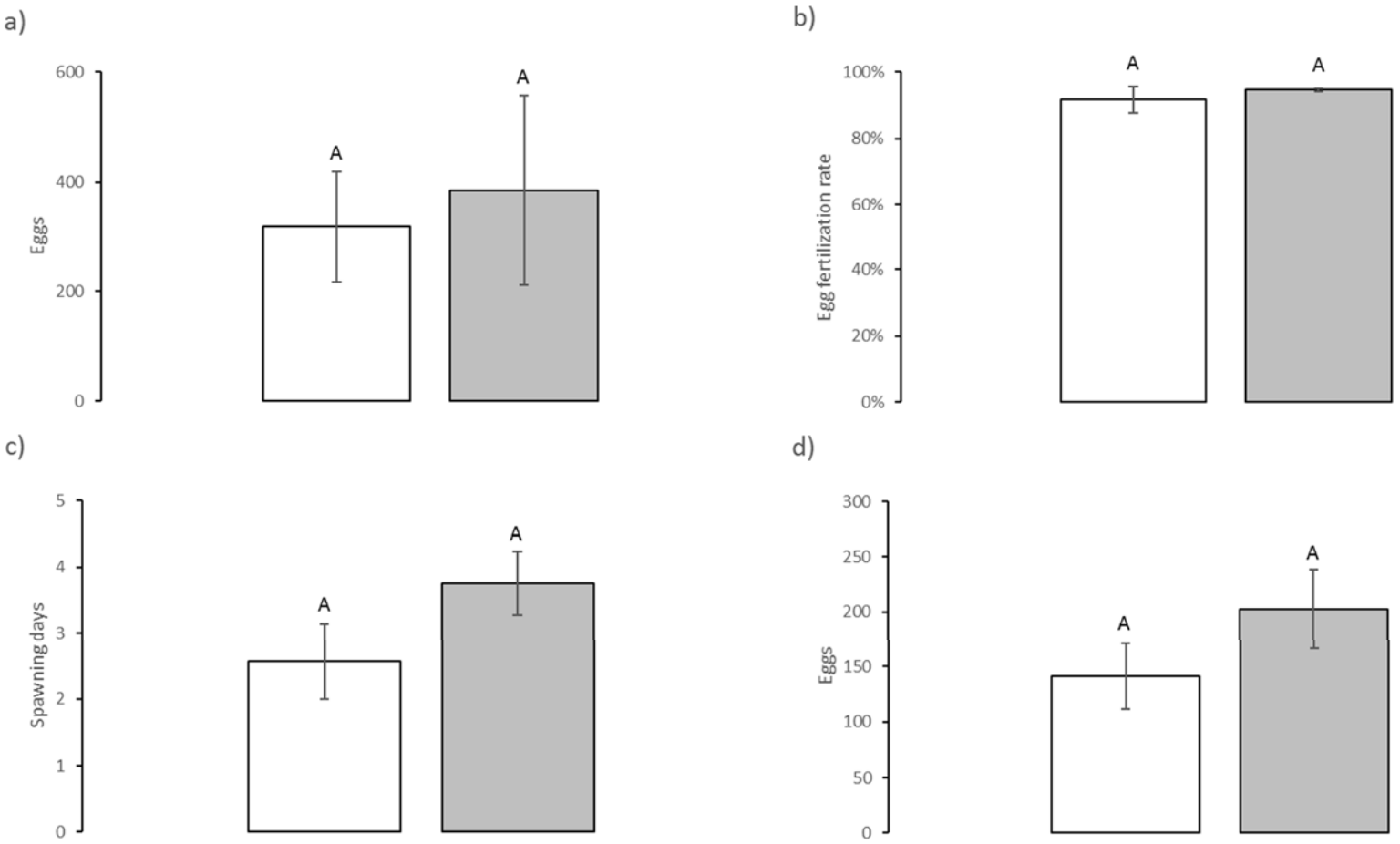

Figure 5-6. Mean (±SE) egg-based endpoints for ps4. Minnows (n = 7 pairs for the tap water treatment, 4 pairs for the 1000 ppm treatment) were acclimated for a 14-week period to tap water (white bars) or 1000 ppm salinity (gray bars), and then tested in corresponding salinity for a 3-week trial. Data recorded includes: a) total eggs produced per pair, b) percent fertilization, c) number of days when spawning took place, and d) clutch size. Different capital letters above the error bars indicate significant differences at alpha of 0.05.

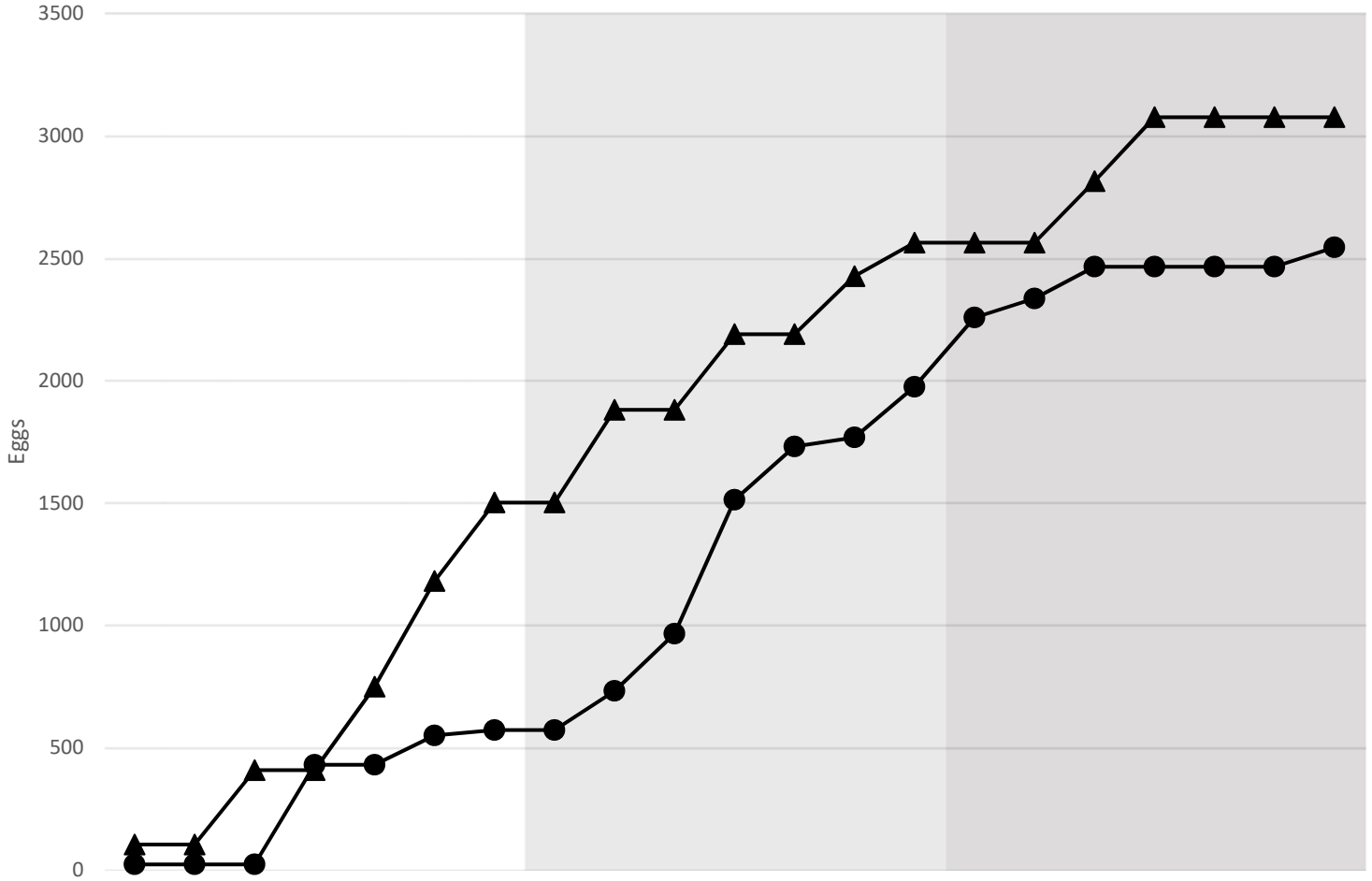

Figure 5-7. Cumulative eggs produced during ps4. Total number of eggs produced for each treatment (tap water represented by circles, n = 7 pairs; 1000 ppm represented by triangles, n = 4 pairs) over the 3-week trial (week 1 = white, week 2 = light gray, week 3 = dark gray).

5.5 Discussion

This study was designed to 1) determine the acclimation point at which minnows held in 1000 ppm salinity would match reproductive endpoints of minnows held in dechlorinated tap water, and 2) determine the effects of changing salinity, if any, on fish acclimated to 1000 ppm. I believed an acclimation point would likely be found, given that fathead minnows are present in Saskatchewan water bodies with salinities up to 10,000 ppm (Rawson & Moore, 1944). After fourteen weeks of exposure I found no significant differences in egg-based fecundity measures (total eggs produced per pair, percent fertilization, number of spawning days, and average clutch size) between control fish and those exposed to 1000 ppm salinity. Therefore, I believe I have found a suitable acclimation point for this salinity level. However, it must be noted that my scope of inference may be limited by my reduced sample size (control n = 7, 1000 ppm n = 4 pairs). It must also be noted that these missing pairs were removed because they did not produce any eggs during the three-week trial. It is unknown why these pairs did not produce eggs. Because the fish were acquired when fully mature, perhaps several pairs had reached the end of their natural reproductive period, or perhaps being held in a water temperature conducive to reproductive state with no mates present in the holding tanks may have affected the fish.

Pilot study 2 (six-week exposure) proceeded as predicted until day 11 of 21 (Figure 5-5). At this point, all control pairs stopped producing eggs, and did not produce any more throughout the subsequent ten days of the trial. Interestingly, no pairs in either treatment produced eggs in pilot study 3 (ten-week exposure). The cause of this disruption in egg production is unknown. It was suggested in an advisory committee meeting that perhaps the seals on the recirculating pumps may have worn, allowing pump oil to leak into the experimental water, thus affecting reproductive output. This seems a plausible explanation, though no pumps failed during the study. If pump oil did leak, the contaminated water would have been removed during the biweekly complete-system water changes (5.3.3 Salinity preparation), three of which occurred before pilot study 4 began. Alternatively, there may have been some unknown contamination of the dechlorinated source water system/storage tanks, or within the recirculating systems themselves, which may have negatively affected reproductive output. However, this seems less likely than pump seal failure, given that the 1000 ppm treatment continued producing eggs throughout pilot study 2 while producing no eggs during pilot study 3. I did not perform another pilot study after pilot study 4 because of logistical constraints—several mortalities occurred in the minnow holding tanks during the four pilot studies, limiting the number of fish available for subsequent

studies. It seems possible that these mortalities may be linked to stress caused from holding minnows in reproductive-state inducing conditions for several months.

After determining an appropriate acclimation period, I intended to expose 1000 ppm acclimated minnows to different salinity concentrations (250, 1000, 4000, and 8000 ppm), using a short-term reproduction assay, which would have included both pre-exposure and exposure phases. Having reached an acclimation point, I proceeded with the assay. However, only 2 out of 32 tanks produced eggs in the pre-exposure phase. I allowed the fish to rest for one month, returning them to holding tanks, however, too many mortalities occurred during this period to continue the experiment. Without adequate numbers of fish to attempt another assay, the experiment was concluded.

Increasing salinity poses a threat to many inland water bodies, and it has been shown that sub-lethal increases in salinity can negatively impact reproduction in fathead minnows (Chapter 4). This acclimation study aimed to determine whether these negative impacts could be overcome by acclimation to low-level salinity, and if this acclimation might affect how minnows responded to further changes in salinity. I was able to determine an appropriate acclimation point. Unfortunately, I was not able to expose acclimated minnows to further changes in salinity. Because inland water bodies of the North American Great Plains are predicted to experience increased salinization in the future (Covich et al., 1997), and because the loss of fathead minnows is associated with alterations of many characteristics of small lakes across the same region (Zimmer et al., 2002), I feel that this experiment should be repeated. I believe this experiment has the potential to help us understand how fathead minnows might respond to future salinity challenges, and how this response may impact populations across the region. I feel that the approach taken in this study is valid, though perhaps fish should be held in lower temperatures during acclimation periods to reduce stress, and slowly brought to reproductive state as trials approach. Additionally, it may be worthwhile to source pumps which cannot leak oil into the experimental water.

Chapter 6: Study 5—Impact of salinity on second-generation fathead minnows (*Pimephales promelas*)

This study contains unpublished data.

6.1 Abstract

Salinization is a challenge facing many inland aquatic systems. Stenohaline fishes facing this challenge must accommodate changes in salinity or face potentially serious consequences. In addition to survival, fishes must also undertake important activities such as foraging, reproduction, and avoiding predators; these activities may be impacted at sub-lethal salinity concentrations. Few studies have explored sub-lethal impacts on fish behaviour, and to my knowledge, no studies have examined the generational effects of sub-lethal salinity exposure on stenohaline freshwater fishes. Here, I spawned, hatched, and reared fathead minnows (*Pimephales promelas*) in two different salinities, dechlorinated tap water (control) and 1000 ppm salinity. At 6-7 months' age, I examined morphological differences between the groups. I found a significant difference, but this was due to rearing tank, not salinity. Next I exposed minnows in each salinity group to one of two alarm cue treatments (deionized water or alarm cue) to examine antipredator behaviour. I found no difference between salinity treatments for antipredator behaviour, suggesting that the predator responses of second-generation minnows are not affected by long-term exposure to moderate salinity.

6.2 Introduction

Salinization of inland water bodies is a concern facing many aquatic animals. Because freshwater organisms facing this problem often lack physiological mechanisms to overcome abrupt changes in salinity, much research focuses on survival. Fewer studies have examined the impacts of sub-lethal salinity on important behaviours. These behaviours can be complex and nuanced. For example, foraging, reproduction, and antipredator behaviours often depend on multiple cues and pressures. The effects of acute, short-term salinity exposure on each of these behaviours has been studied in freshwater stenohaline fishes, but only in a few species. For example, when exposed to salinity, feeding behaviour has been reduced in goldfish (*Carassius auratus*) (Luz et al., 2008), reproductive output and behaviour has been negatively impacted in fathead minnows (*Pimephales promelas*) (Hoover, Weisgerber, et al., 2013), and antipredator responses have been reduced in fathead minnows as well (Hoover, Ferrari, et al., 2013). However, to my knowledge, no studies have examined the generational effects of salinity exposure on stenohaline freshwater fishes.

Another limitation to previous research is that the vast majority of studies only consider the effects of NaCl. While this is likely due to the widespread abundance of NaCl dominated inland waters

around the world, it is important to consider the effects of other dominant ions. In fact, other major ions, common to many inland waters (such as MgSO4 dominated lakes in Saskatchewan), may have even more dramatic effects than NaCl. For instance, Mount et al. (1997) found that the 96-h LC50 for fathead minnows varied from <510 to 7960 parts per million (ppm) depending on the ion ratio and salts present in the experimental water, with the following relative ion toxicity: $K^+ > HCO_3^- \approx Mg^{2+} > Cl^- > SO_4^{2-}$. Similarly, for rainbow trout (*Oncorhynchus mykiss*) and larval chironomids (*Chironomus tentans*), Chapman et al. (2000) found the toxicity of mining effluents was not predictable from total dissolved solids (TDS) concentration alone, but instead depended on the specific combination and concentration of ions.

Here, I spawned, hatched, and reared fathead minnows (*Pimephales promelas*) in two different salinities, dechlorinated tap water (control) and 1000 ppm salinity. At 6-7 months' age, I examined morphological differences between the groups. Next, I exposed minnows in each salinity group to one of two alarm cue treatments (deionized water or alarm cue) to examine antipredator behaviour. Because acute exposure to 1000 ppm salinity has been shown to affect reproductive output in fathead minnows, I believed there was a possibility it might also affect the morphology and antipredator behaviour of minnows spawned, hatched, and reared in 1000 ppm saline water.

6.3 Methods

6.3.1 Experimental design

Fish were spawned, hatched, and reared in either dechlorinated tap water (control) or 1000 ppm salinity. At age 6-7 months, fish were compared for morphological differences (N = 84, tap water n = 40, 1000 ppm n = 44) then tested for antipredator responses using a fully randomized 2x2 design.

6.3.2 Experimental fish

Fathead minnows were spawned, hatched, and reared in the lab (Study 4—Impact of salinity change on acclimated fathead minnow (*Pimephales promelas*) reproduction). Before hatching, minnow eggs were housed in 2 L glass aquaria equipped with an air stone, and filled with water matching the

salinity in which they were spawned. Two days after hatching, minnow larvae were fed brine shrimp nauplii (*Artemia sp.*) *ad libitum* which were produced in the lab from cysts (Brine Shrimp Direct, Ogden, UT, USA). Daily water changes, with removal of uneaten nauplii, were performed to avoid fouling the water. Fish were also fed crushed commercial fish flakes (Nutrafin Max Flake Food, Rolf C. Hagen Inc., Montreal, QC) once they began showing interest. Once deemed too large to escape rearing tanks, fish were moved to a recirculating system (Figure 5-2). Rearing tanks were 37 L glass aquaria equipped with an air stone and a gravel substrate. At this point, fish were no longer fed brine shrimp. Laboratory conditions were maintained at ≈ 25° C for optimum growth (OECD, 2009; USEPA, 1987, 2002), with a 16:8 h light:dark photoperiod and at least 80% oxygen saturation. Upon maturation, room temperature was lowered to approximately 20° C so that minnows would not enter reproductive state, as minnows in reproductive state may show reduced antipredator responses (Pollock et al., 2006).

6.3.3 Stimulus collection

Alarm cues were prepared using 15 fathead minnows (mean ± SD: fork length 4.9 ± 0.4 cm; weight 1.7 ± 0.4 g) following the method described in Ferrari et al. (2005). The minnows were euthanized by cervical dislocation, in accordance with University of Saskatchewan Animal Care protocol #20100023. Skin fillets were removed from each side of the body and immediately placed in 100 ml of chilled distilled water. The skin solution was then homogenized and filtered through glass wool. This procedure resulted in 24.7 cm^2 of skin in 494 ml of distilled water to give a stock solution of 1 cm^2 of skin per 20 ml. This stock solution was then serially diluted to obtain alarm cue solutions (concentration = 1 cm^2/40 L). These solutions, along with distilled water, were frozen in 20 ml aliquots at -20°C until use.

6.3.4 Salinity preparation

Experimental water was prepared by adding the salts listed in Section 4.3.3 Salinity preparation to dechlorinated tap water. See Figure 6-1 for a representation of the milligram equivalent per liter (mEq/L) percent ion composition of the experimental water.

Experimental water was prepared by adding the salts and dechlorinated tap water to mixing tanks (150 L volume). The solutions were then circulated within the mixing tanks overnight. This approach aided in the dissolution of salts, and also aided in bringing each solution to room temperature.

Control water followed the same procedures as saline water. The rearing tanks utilized a recirculating system. Therefore, mixing tanks holding experimental water were replenished daily with dechlorinated tap water in order to counteract losses due to evaporation. Additionally, all water was flushed from the system and new water was prepared every two weeks. Experimental tanks were filled with water from the recirculating system, and were emptied into the sewer after trials.

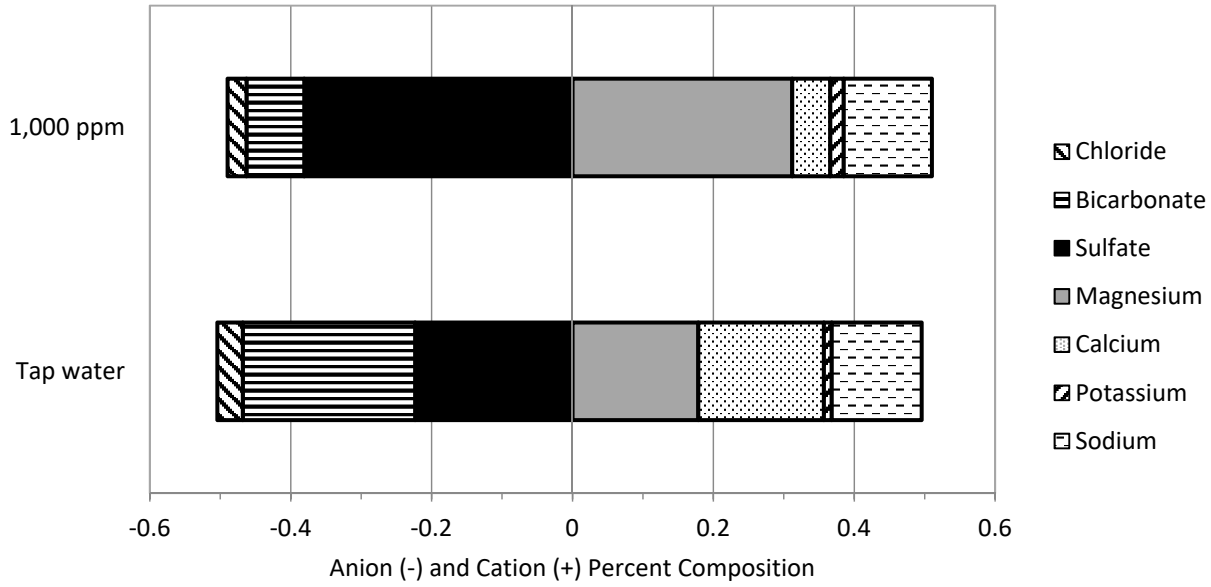

Figure 6-1. Average milligram equivalent per liter ion composition for control and experimental waters for Study 5. The ion composition of experimental waters is based on sampling of Lake Lenore by the Saskatchewan Watershed Authority (2007–2008). Values are based on the results of independent laboratory analysis (Saskatchewan Research Council, Saskatoon, Canada). Ions are divided into anions and cations. Note that percent composition should be considered an absolute value; anions are represented as negative numbers to facilitate comparison.

6.3.5 Test apparatus and acclimation period

A single minnow was placed in each experimental tank (N = 84). Experimental tanks consisted of 9 L plastic aquaria equipped with an air stone, a stimulus injection tube, a lid, and a shelter object (10 cm diameter PVC pipe cut to a height of 2 cm, 7.5 cm long). Shelters were shorter than those used in Study 3 (Section 3.3.5 Test apparatus and acclimation period) in an attempt to reduce pre-exposure shelter use when compared to Study 3. Additionally, a 3x3 grid was marked on each tank to determine line crosses during trials. Experimental tanks were also blacked out on three sides to prevent visual cues from adjacent tanks. Fish were given 72 h to acclimate to experimental tanks before trials began.

6.3.6 Testing procedure

Testing followed the procedure outlined in Section 3.3.6 Testing procedure. In addition to the measures described in Section 3.3.6 Testing procedure, I also recorded the number of times fish crossed the lines drawn on the outside of the experimental tanks. At the conclusion of the trial, water quality parameters (conductivity, pH, dissolved oxygen, and temperature) were recorded for each tank with a YSI probe (Professional Plus, YSI Inc., Yellow Springs, OH). Additionally, 25 ml of tank water was withdrawn from each tank and combined by treatment group for analysis by an independent laboratory (Saskatchewan Research Council, Saskatoon, Canada) for verification of ion composition and salinity concentration (sum of ions). See Figure 6-1 and Table 6-1.

Table 6-1. Mean (± SD) water quality parameters. Percent error for TDS concentration is based on comparison with nominal theoretical values. The negative value for the 8,000 ppm group denotes the slightly lower than theoretical value.

Treatment	Tap water	1000 ppm
Conductivity (µS/cm)	341 (26)	1095 (31)
Dissolved Oxygen (%)	84.3 (6.9)	85.4 (6.6)
pH (pH units)	7.9 (0.1)	7.9 (0.1)
Temperature (°C)	19 (1)	19 (1)
Sum of ions (ppm)	286	970
% Error	---	-3%

6.3.7 Statistical analyses

Statistical analyses were performed with R version 3.2.3 (R Development Core Team, 2015), using the RStudio IDE, version 0.99.491 (RStudio Team, 2015). All data were checked for conformance to test assumptions (normality and homogeneity of variance) using R's built-in diagnostic plots and the Fligner-Killeen test for homogeneity of variance. Data were transformed as noted below when those assumptions were not met.

6.3.7.1 Morphological comparison and assignment to experimental treatment groups

Standard length and weight of each fish was measured before randomly assigned to an experimental tank. A 1-way MANOVA was performed to determine whether there were any morphological differences between fish raised in dechlorinated tap water and those raised in 1000 ppm salinity. Morphological data did not meet test assumptions, and were log transformed. Separate ANOVAs were then performed to determine differences between groups. Groups differed by length and weight, therefore regression was performed on length and weight, and the residuals were analyzed by salinity concentration and rearing tank, separately, in order to determine the cause of the observed differences.

A 2x2 MANOVA was performed on the morphological data of fish assigned to each treatment group (salinity x alarm cue treatment) to determine any differences among groups. Individual ANOVAs were then performed to determine differences among groups for length and weight. Significant differences were evaluated with Tukey's HSD.

6.3.7.2 Water quality

Water quality parameters (conductivity, dissolved oxygen, pH, and temperature) were compared between salinity treatments using a 1-way MANOVA. Separate ANOVAs were then performed to investigate which of the parameters differed significantly among levels. Temperature was log transformed to meet test assumptions.

6.3.7.3 Behavioural data

Pre-stimulus data (number of line crosses, time spent moving, and shelter use) were heteroscedastic. No adequate transformations were found, so Mann-Whitney U tests were performed.

Percent change (post-stimulus – pre-stimulus / pre-stimulus) was used as a measure of antipredator behaviour for line crosses and time spent moving. Because pre-stimulus shelter use was often 0 s, percent change in shelter use was not possible to calculate. Instead, change in shelter (pre-stimulus – post-stimulus) was used. A 2-way ANOVA was performed for each measure of antipredator behaviour to determine any differences among treatment groups. None of the measures met test assumptions. All antipredator measures were unbounded and also contained both positive and negative numbers, therefore data were rank transformed.

6.4 Results

6.4.1 Morphological comparison and assignment to experimental treatment groups

Minnows spawned, hatched, and reared in 1000 ppm salinity were longer (standard length mean ± SD: 3.2 ± 0.3 cm) and had more mass (0.6 ± 0.2 g) than control dechlorinated tap water fish (length = 2.8 ± 0.5 cm, weight = 0.4 ± 0.4 g), (Pillai's Trace: $F_{(1,82)} = 8.7$, $p < 0.001$; $ANOVA_{length}$: $F_{(1,82)} = 15$,

$p < 0.001$; $ANOVA_{weight}$: $F_{(1,82)} = 17$, $p < 0.001$; Table 6-2). However, analysis of residuals revealed that rearing tank was responsible for these differences ($F_{(3,80)} = 5.4$, $p = 0.002$), not salinity treatment ($F_{(3,80)} = 2.0$, $p = 0.16$). See Table 6-3 for a summary of physical characteristics for each rearing tank.

Table 6-2. Mean (± SD) physical characteristics by rearing salinity for Study 5.

Rearing salinity	Tap water	1000 ppm
Length (cm)	2.8 (0.5)	3.2 (0.3)
Weight (g)	0.4 (0.4)	0.6 (0.2)

Table 6-3. Mean (± SD) physical characteristics by rearing tank for Study 5. 7 = July (hatch month), 8 = August, TW = tap water, 1000 = 1000 ppm.

Rearing tank	7.TW	8.TW	7.1000	8.1000
Length (cm)	2.7 (0.3)	3.8 (0.4)	3.1 (0.4)	3.3 (0.2)
Weight (g)	0.3 (0.1)	1.3 (0.2)	0.5 (0.2)	0.6 (0.1)

A significant difference was found among treatment groups for salinity treatment (Pillai's Trace, salinity: $F_{(1,80)} = 8.5$, $p < 0.001$; cue: $F_{(1,80)} = 0.57$, $p = 0.57$; interaction: $F_{(1,80)} = 0.02$, $p = 0.98$; Table 6-4). Individual ANOVAs found no difference among treatment groups for weight ($F_{(3,80)} = 1.7$, $p = 0.17$). However, there was a significant difference among treatment groups for standard length ($F_{(3,80)} = 4.3$, $p = 0.007$), with fish in the 1000 ppm alarm cue-exposed group being longer than fish in the dechlorinated tap water water-exposed group (Tukey comparison: $p = 0.02$; Table 6-4). No other significant differences were found.

Table 6-4. Mean (± SD) physical characteristics by treatment group for Study 5. TW = tap water, 1000 = 1000 ppm, W = deionized water cue, AC = alarm cue.

Treatment group	TW.W	TW.AC	1000.W	1000.AC
Length (cm)	2.8 (0.4)	2.9 (0.4)	3.1 (0.4)	3.2 (0.4)
Weight (g)	0.5 (0.2)	0.4 (0.3)	0.6 (0.3)	0.6 (0.2)

6.4.2 Water quality

A significant difference was found between salinity treatment groups (Pillai's Trace: $F_{(1,82)} = 4346$, $p < 0.001$; Table 6-5). As predicted, conductivity was different between groups ($F_{(1,82)} = 13066$, $p < 0.001$), while dissolved oxygen ($F_{(1,82)} = 0.6$, $p = 0.45$), pH ($F_{(1,82)} = 3.8$, $p = 0.06$), and temperature ($F_{(1,82)} = 1.1$, $p = 0.30$) did not differ.

Table 6-5. Mean (± SD) water quality parameters for Study 5. Percent error for sum of ions is based on the actual sum compared to nominal theoretical value.

Treatment	Tap water	1000 ppm
Conductivity (µS/cm)	341 (26)	1095 (31)
Dissolved Oxygen (%)	84.3 (6.9)	85.4 (6.6)
pH (pH units)	7.9 (0.1)	7.9 (0.1)
Temperature (°C)	19 (1)	19 (1)
Sum of ions (ppm)	286	970
% Error	---	-3%

6.4.3 Behavioural data

Pre-stimulus behaviour did not differ among treatment groups for line crosses (Mann-Whitney U test: U = 900, $p = 0.68$), time spent moving (U = 864, $p = 0.89$), or shelter use (U = 875, $p = 0.97$). See Figure 6-2.

Fish exposed to alarm cues showed a significantly different percent change in number of line crosses than those exposed to deionized water, but their response did not differ between salinity treatments (2-way ANOVA: cue: $F_{(1,80)} = 14$, $p < 0.001$; salinity: $F_{(1,80)} = 0.36$, $p = 0.55$; interaction: $F_{(1,80)} = 0.07$, $p = 0.79$; Figure 6-3a). Similarly, percent change in movement was significantly affected by alarm cue exposure, but not salinity level (cue: $F_{(1,80)} = 104$, $p < 0.001$; salinity: $F_{(1,80)} = 0.44$, $p = 0.51$; interaction: $F_{(1,80)} = 1.7$, $p = 0.20$; Figure 6-3b). Change in shelter use was not affected by cue exposure or salinity level (cue: $F_{(1,80)} = 3.5$, $p = 0.06$; salinity: $F_{(1,80)} = 0.27$, $p = 0.61$; interaction: $F_{(1,80)} = 0.00$, $p = 0.99$; Figure 6-3c).

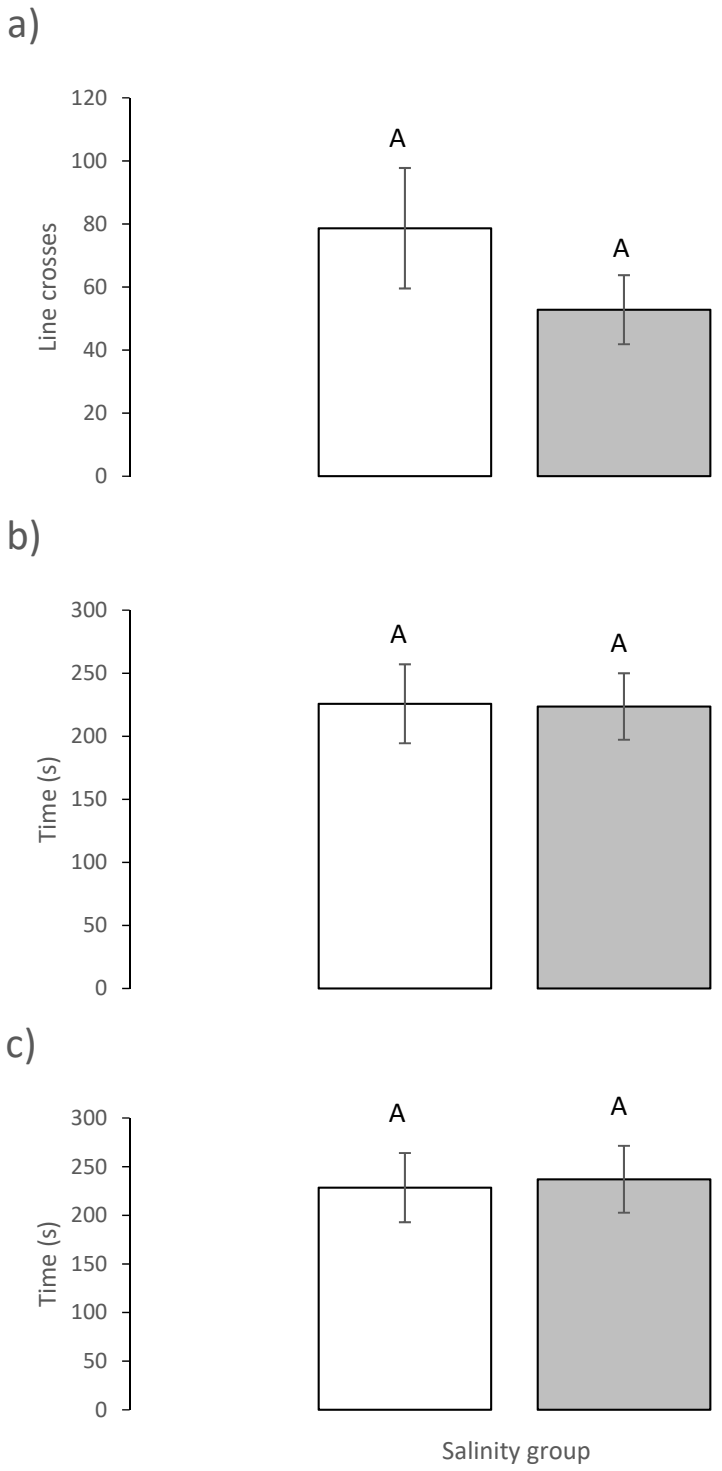

Figure 6-2. Mean (± SE) pre-stimulus data for Study 5. Minnows (n = 40 control tap water [white bars], n = 44 1000 ppm salinity [grey bars]) were observed for an 8-min pre-stimulus period, and data for a) number of lines crossed, b) time spent moving, and c) time spent under shelter were recorded for each group. Different letters above the error bars indicate significant differences at alpha of 0.05.

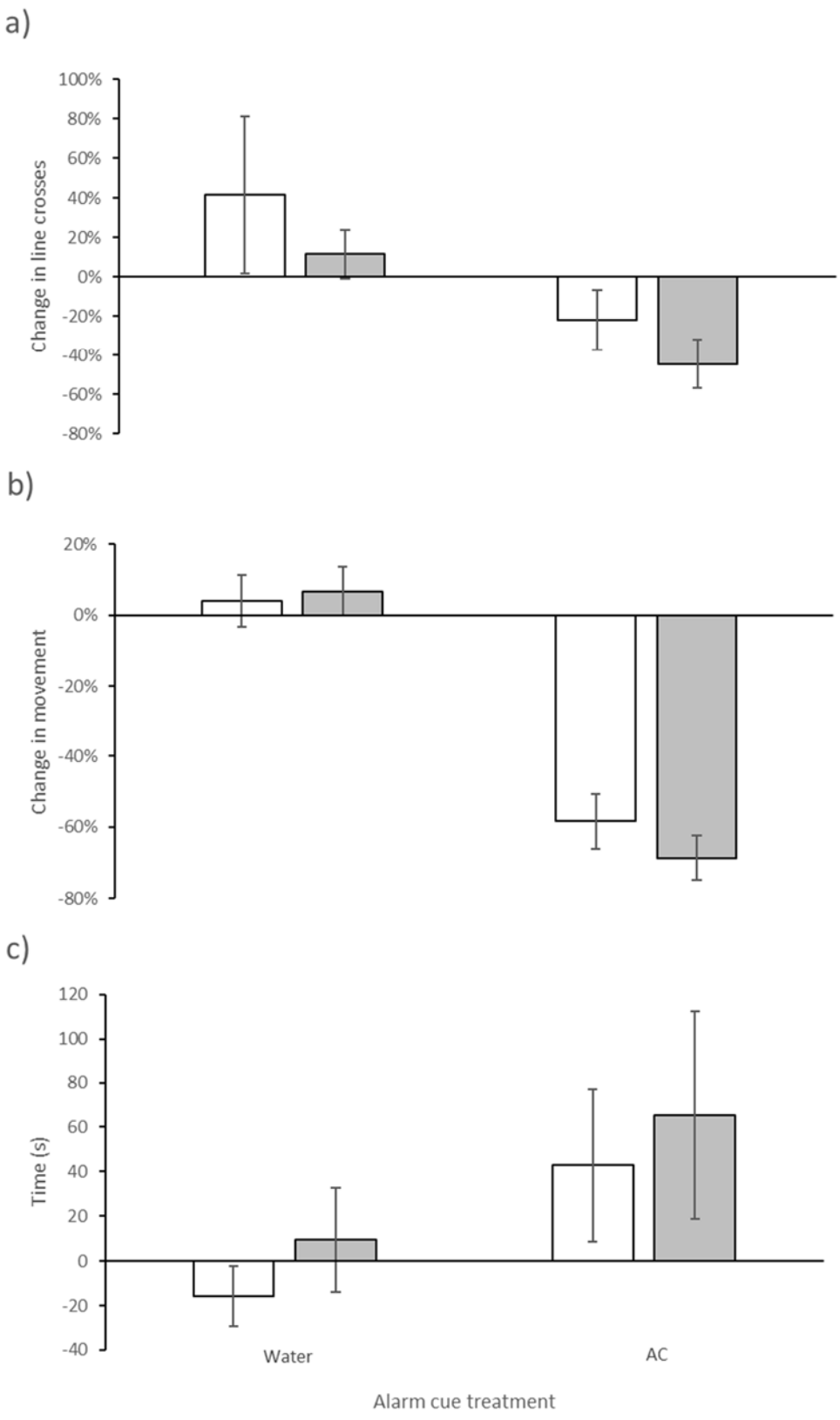

Figure 6-3. Post-stimulus data for Study 5. Mean (± SE) a) percent change in line crosses, b) percent change in movement, and c) change in shelter use when fathead minnows (N = 84) were exposed to either deionized water or alarm cues while being maintained in either tap water (white bars) or 1000 ppm salinity (grey bars).

6.5 Discussion

For this study, I was interested in 1) whether the morphological characteristics of fathead minnows spawned, hatched, and reared in low-level saline water (1000 ppm) would differ from minnows spawned, hatched, and reared in dechlorinated tap water, and 2) whether these two groups would show any significantly different response to predation cues. To this end, I produced several groups of minnows (aged 6-7 months at the time of testing). I originally intended to include other salinity groups of 4000 and 8000 ppm, but could not generate the numbers required for this study.

I found that fish from the 1000 ppm group were longer and had more mass than fish in the control group. However, upon closer examination, I found that these differences were due to rearing tank, not salinity treatment. The smallest minnows, which were hatched in dechlorinated tap water during the month of July, had the highest tank density (approximately 70 fish). Moreover, the largest minnows, which were hatched in dechlorinated tap water during the month of August, had the lowest tank density (12 fish at the beginning of the study). Fish hatched in 1000 ppm salinity in the month of July and August were reared in tanks with 26 and 20 fish, respectively. Because the rearing tanks were on a recirculating system with a constant flow of water, I did not anticipate finding significant differences between tap water groups when rearing began. However, this seems to have been an incorrect assumption. Ideally I would have split the July tap water group into multiple tanks, but I did not.

I also found a significant difference among minnows assigned to treatment groups, with the tap water bred, water-exposed group being significantly shorter than the 1000 ppm alarm cue-exposed group (mean ± SD: 2.8 ± 0.4 and 3.2 ± 0.4 cm, respectively). It should be noted, however, that this difference in length is small (0.4 cm), and no other treatments were significantly different, including the tap water and 1000 ppm groups which were exposed to alarm cues. Therefore, I feel that this difference, though technically significant, probably played very little role in my results.

Typical antipredator responses in fathead minnows include a reduction in movement and an increase in shelter use. I found a significant difference in percent change for line crosses and total time spent moving between alarm cue treatments. However, I found no effect between salinity treatments. Therefore, I can reasonably infer that the movement of minnows spawned, hatched, and reared in a salinity concentration of 1000 ppm and exposed to alarm cues is not significantly affected when compared to tap water controls. I did not, however, find a significant effect of alarm cue treatment on

shelter use. In a previous experiment (Study 2—Impact of salinity on fathead minnow (*Pimephales promelas*) antipredator responses), we asserted that the shelters we used were too large for the experimental tanks. Therefore, I reduced shelter size for this study. This change in size did reduce shelter use relative to the previous experiment, but pre-stimulus shelter use remained rather high (approximately 49% of pre-stimulus observation time). Though I can find a significant effect of alarm cue treatment on change in shelter use by removing fish with high pre-stimulus shelter use, this approach would reduce my sample size from 84 to 44, so I do not feel such removals to be warranted. Additionally, shortening the shelters beyond the height of those used in this study would result in shelters which were physically inaccessible to some of the minnows. Therefore, perhaps it is not possible to provide adequate shelters for fathead minnows in 9 L experimental tanks. Ideally, I would have used larger tanks, but preparing experimental water and space for 84 37 L tanks was logistically prohibitive. Alternately, I originally planned on using trios, as opposed to single fish in every tank, so that shelters would not be necessary, but I did not generate enough fish for this approach.

As originally envisioned, comparing morphological characteristics and antipredator behaviour across multiple salinities, this study may have generated useful data to help estimate the generational impacts of salinization on fathead minnows over time. Despite the logistical challenges faced in this study, I still managed to demonstrate that fathead minnows should be able to maintain normal behaviours and characteristics with modest long-term increases in salinity, especially when considered with the results of Study 4 (Section 5.5 Discussion). However, it is still important to consider how more dramatic changes in salinity may affect fathead minnows. Therefore, I believe it would be worthwhile to reproduce this study with multiple salinity levels, though it may be logistically difficult.

Chapter 7: Discussion

7.1 Salinity and survival

The majority of work produced for this thesis examines the effects of sub-lethal salinity exposure on fishes. However, before we can investigate the effects of sub-lethal salinity, we must first determine lethal levels. Several studies have investigated the lethal effects of salinity on freshwater fishes by performing LC50s (for example, Jacobsen et al., 2007; Mojazi Amiri et al., 2009; Whiterod & Walker, 2006), but the vast majority of studies only consider the effects of NaCl. As previously stated, it is important to consider the effects of other dominant ions, given that Mount et al. (1997) found that the 96-h LC50 for fathead minnows varied from <510 to 7960 ppm depending on the ion ratio and salts present in the experimental water, with the following relative ion toxicity: $K^+ > HCO_3^- \approx Mg^{2+} > Cl^- > SO_4^{2-}$. Because saline water bodies in Saskatchewan tend to be dominated by $MgSO_4$ and Na_2SO_4 (Hammer, 1978b), results from previous studies were not directly applicable to our species of interest.

Given the limitations of our LC50 studies (no concentrations between 8000 ppm and 16000 ppm tested; no mortalities in the 8000 treatment, 100% mortality in the 16000), our results for fathead minnows match the existing literature fairly well. Our LC50 value of 11627 ppm for minnows compares favourably with Ingersoll et al. (1992) who found an acute toxicity range of 8000-10000 ppm (no precise LC50 value reported) for NaCl. However, it must be mentioned that the minnows used in the Ingersoll study were only two days post-hatch, and the researchers did not use a slow-acclimation approach, which has been shown to reduce stress in freshwater fishes (Kefford et al., 2004; Whiterod & Walker, 2006). Both of these conditions, small body size and no acclimation period, potentially impacted their estimate. Our results also compare favourably with the surveys performed by Rawson and Moore (1944) which found fathead minnows in saline lakes with a salinity concentration of approximately 10000 ppm. These surveys have direct relevance to our LC50, as our experimental water was designed to have a similar ion makeup to the water bodies included in the surveys.

Our results also compare favourably with Jacobsen et al. (2007). Our estimated LC50 value of 11627 ppm is reasonably close to the Jacobsen range of 12000-14000 ppm. The Jacobsen study reports a range because they used two body sizes and three temperatures. However, as with comparing our minnow data to the Ingersoll study, direct comparisons with the Jacobsen study are not recommended because, in addition to using different major ions, the Jacobsen study also used smaller fish and did not use a slow-acclimation approach. Interestingly, our pike LC50 value is also contradicted by two

Saskatchewan surveys in the literature. First, Rawson and Moore (1944) report that another survey conducted by Huntsman in 1922 found northern pike in Big Quill Lake with a salinity concentration of approximately 16550 ppm. Then, confusingly, Rawson and Moore also noted that northern pike were killed in Lake Lenore when salinity reached 6034 ppm. This rather low finding might be explained by possible confounding factors, should they exist, such as extreme temperature or pollution of the water. However, the Rawson and Moore survey of Lake Lenore found that pike were the only species of fish (out of eight) killed by the rise in salinity.

Little data exists on the salinity tolerance of walleye. Perhaps this is related to the apparent difficulty of maintaining walleye in a laboratory setting. As noted in Chapter 2, the walleye used for this study were hesitant to feed, and this resulted in low body mass. Given this low body mass, it is unsurprising to find that our LC50 estimate of 8316 ppm is quite low when compared to Rawson and Moore's surveys, which found walleye in numerous lakes with salinity concentrations of 10000-15000 ppm.

7.2 Salinity and predation

The complex antipredator behaviour of fathead minnows has been well-characterized, and our research shows that this behaviour can be modified by exposure to salinity. To our knowledge, the data from Chapter 3 are the first to show an effect of salinity on the antipredator behaviour of fathead minnows. We were also able to show a reduction in movement after exposure to salinity. In Chapter 2, we found a reduction in movement at 6000 and 8000 ppm. This is consistent with our results in Chapter 3, where we found a pre-stimulus reduction in movement at 8000 ppm. Similarly, Whiterod and Walker (2006) found a reduction in movement in common carp at 5000 ppm, though the scale used by those researchers was rather subjective (a six-point scale with 5 = normal movement and 0 = no movement). Interestingly, Luz et al. (2008) found that goldfish reduced movement in salinity concentrations as low as 2000 ppm. This concentration seems rather low, though it is based on 12-hour daily observations over a 21-day period using automated recorders. Perhaps minnows would show a similar reduction in movement at lower concentrations if observations and exposure periods were longer.

Unsurprisingly, we found that alarm cues elicited fathead minnow antipredator responses in Chapters 3 and 6, in agreement with virtually all the relevant literature. Interestingly, we found a decrease in the intensity of response for minnows exposed to 8000 ppm salinity in Chapter 3. As

mentioned in Chapter 3, this reduction could possibly be due to either 1) improper function of the olfactory system based on interactions with ions dissolved in the water, at the alarm cue or receptor level; or (in my opinion more likely) 2) increased metabolic costs of osmoregulation in the saline environment.

In agreement with Helfman's threat-sensitive hypothesis, we found a threat-sensitive response to alarm cues in the 1000 ppm salinity treatment in Chapter 3. However, this response was absent in the 4000 and 8000 ppm treatment groups, suggesting threat-sensitivity may be a more sensitive measure than overall antipredator response. Additionally, this lack of threat-sensitive response may have fitness consequences for minnows experiencing a rather moderate increase in salinity (Lima & Dill, 1990).

7.3 Salinity and reproduction

In Chapter 4, I presented the results of our investigation into the effects of salinity on fathead minnow reproductive endpoints. To my knowledge, we were the first to examine this issue in fathead minnows. Of the egg-based endpoints we measured (average total eggs produced per pair, percent fertilization, number of spawns, and clutch size), total eggs produced per pair seemed the most sensitive, and percent fertilization the least. We found that percent fertilization was only affected in the highest salinity treatment—8000 ppm. This is somewhat surprising, given that fertilization rate can be impacted in salmonids at concentrations as low as 250 ppm (Stekoll et al., 2009). However, it is important to note that Rosengrave et al. (2009) demonstrated that sperm longevity in chinook salmon was negatively correlated with Ca^{2+} and Mg^{2+} concentrations in the ovarian fluid surrounding eggs. It is likely that fathead minnow sperm longevity is similarly affected by saline water with high concentrations of these ions. Given that the concentration of Ca^{2+} and Mg^{2+} in the 8000 ppm treatment was roughly double that of the 4000 ppm treatment, this seems a likely explanation for the observed reduction in fertilization success in the 8000 ppm treatment.

We also demonstrated an impact of salinity on male fathead minnow reproductive behaviour in Chapter 4, specifically time spent in nest care and duration of nest care events in the 8000 ppm treatment. Nest care by the male is important, as it keeps eggs clear of fungus and is linked to protection of the eggs, which would otherwise be eaten by the female or another male (McMillan & Smith, 1974; Weber & Bannerman, 2004).

In Chapter 5, I was able to demonstrate an adequate acclimation period of 14 weeks. After this period, fish held in 1000 ppm salinity matched reproductive endpoints with fish held for the same amount of time in dechlorinated tap water. However, it is important to note that my sample size was reduced from eight to seven in the tap water treatment, and from eight to four in the 1000 ppm treatment, thus potentially limiting my scope of inference.

7.4 Conclusion

With virtual certainty that global temperatures will increase in the near future (IPCC, 2014), it follows that endorheic drainage basins, like those found in the Northern Great Plains of North America, will likely see increased salinization. Therefore, it is crucial to understand how salinization may impact aquatic systems in the near future. In this thesis, I have outlined a few of the potential impacts salinization may have on freshwater fishes. In Chapter 2, we demonstrated that, while three common species of freshwater fishes are fairly tolerant to increases in salinity, there is, of course, a limit to their ability to cope. More importantly, we identified sub-lethal concentrations which impact movement in fathead minnows and northern pike. In Chapter 3, we demonstrated that sub-lethal concentrations of salinity can reduce antipredator responses in fathead minnows, including threat-sensitive responses. This effect could potentially, of course, have negative fitness consequences. In Chapter 4, we found that salinity negatively impacted several important egg and behaviour-based endpoints in fathead minnows. The effects were present for total eggs produced per pair even at a relatively low concentration of 1000 ppm. In Chapter 5, I found a potential acclimation point for reproductive endpoints between fathead minnows held in 1000 ppm salinity and those held in dechlorinated tap water. However, I was not able to carry out the further testing I had planned. In Chapter 6, I found no difference in morphology between minnows spawned, hatched, and reared in 1000 ppm salinity and their tap water counterparts. Additionally, I found no difference between the two groups for antipredator behaviour. Unfortunately, I was not able to investigate generational effects at other salinity concentrations.

7.4 Future directions

Though I have answered several questions with the work included in this thesis, many remain. For instance, salinity is unlikely to be the only stressor a freshwater fish will face. A warming climate will,

of course, bring warmer temperatures, but may also bring invasive species and result in increased exploitation and pollution (IPCC, 2014). How will the presence, and potential interaction of these stressors, in addition to salinization, impact aquatic systems? Other interesting questions include: How much phenotypic plasticity exists within a given species to tolerate a range of salinities, and how much variation is found among natural populations? Body size has been shown to impact salinity tolerance. How much advantage might a predator have over a prey fish in a given salinity because of body size? Do stenohaline freshwater fishes actively prefer one salinity over another? How are risk/reward choices impacted by salinity? That is, if faced with a food-rich and dangerous but preferred salinity environment, will a prey fish choose safety in a less-preferred salinity? And, of course, are results from lab-based salinity studies transferrable to real-world environments?

References

Alcaraz, C., Bisazza, A., & García-Berthou, E. (2008). Salinity mediates the competitive interactions between invasive mosquitofish and an endangered fish. *Oecologia, 155*(1), 205-213.

Ankley, G. T., Jensen, K. M., Kahl, M. D., Korte, J. J., & Makynen, E. A. (2001). Description and evaluation of a short-term reproduction test with the fathead minnow (Pimephales promelas). *Environmental Toxicology and Chemistry, 20*(6), 1276-1290.

Ankley, G. T., & Villeneuve, D. L. (2006). The fathead minnow in aquatic toxicology: Past, present and future. *Aquatic Toxicology, 78*(1), 91-102.

Bezirci, G., Akkas, S. B., Rinke, K., Yildirim, F., Kalaylioglu, Z., Severcan, F., & Beklioglu, M. (2012). Impacts of salinity and fish-exuded kairomone on the survival and macromolecular profile of Daphnia pulex. *Ecotoxicology, 21*, 601-614.

Blumstein, D. T., Evans, C. S., & Daniels, J. C. (2006). JWatcher 1.0. Retrieved from http://www.jwatcher.ucla.edu

Bond, N. R., Lake, P. S., & Arthington, A. H. (2008). The impacts of drought on freshwater ecosystems: An Australian perspective. *Hydrobiologia*, 1-14.

Bowman, J. S., & Sachs, J. P. (2008). Chemical and physical properties of some saline lakes in Alberta and Saskatchewan. *Saline Systems, 4*(1).

Brix, K. V., Gerdes, R., Curry, N., Kasper, A., & Grosell, M. (2010). The effects of total dissolved solids on egg fertilization and water hardening in two salmonids-Arctic Grayling (Thymallus arcticus) and Dolly Varden (Salvelinus malma). *Aquatic Toxicology, 97*(2), 109-115.

Brown, G. E., & Smith, R. J. F. (1996). Foraging trade-offs in fathead minnows (Pimephales promelas, Osteichthyes, Cyprinidae): Acquired predator recognition in the absence of an alarm response. *Ethology, 102*(9), 776-785.

Brown, M. G., Dobbs, E. K., Snodgrass, J. W., & Ownby, D. R. (2012). Ameliorative effects of sodium chloride on acute copper toxicity among Cope's gray tree frog (Hyla chrysoscelis) and green frog (Rana clamitans) embryos. *Environmental Toxicology and Chemistry, 31*(4), 836-842.

Cañedo-Argüelles, M., Kefford, B. J., Piscart, C., Prat, N., Schäfer, R. B., & Schulz, C. J. (2013). Salinisation of rivers: An urgent ecological issue. *Environmental Pollution, 173*, 157-167.

Carpenter, S. R., Stanley, E. H., & Vander Zanden, M. J. (2011) State of the world's freshwater ecosystems: Physical, chemical, and biological changes. *Vol. 36* (pp. 75-99).

Chapman, P. M., Bailey, H., & Canaria, E. (2000). Toxicity of total dissolved solids associated with two mine effluents to chironomid larvae and early life stages of rainbow trout. *Environmental Toxicology and Chemistry, 19*(1), 210-214.

Chinathamby, K., Reina, R. D., Bailey, P. C. E., & Lees, B. K. (2006). Effects of salinity on the survival, growth and development of tadpoles of the brown tree frog, Litoria ewingii. *Australian Journal of Zoology, 54*(2), 97-105.

Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signalling in aquatic predator-prey systems: a review and prospectus. *Ecoscience, 5*(3), 338-352.

Chivers, D. P., Wisenden, B. D., Hindman, C. J., Michalak, T. A., Kusch, R. C., Kaminskyj, S. G. W., . . . Mathis, A. (2007). Epidermal 'alarm substance' cells of fishes maintained by non-alarm functions: Possible defence against pathogens, parasites and UVB radiation. *Proceedings of the Royal Society B: Biological Sciences, 274*(1625), 2611-2619.

Cole, K. S., & Smith, R. J. F. (1987). Male courting behaviour in the fathead minnow, Pimephales promelas. *Environmental Biology of Fishes, 18*(3), 235-239.

Cormier, S. M., Suter, G. W., & Zheng, L. (2013). Derivation of a benchmark for freshwater ionic strength. *Environmental Toxicology and Chemistry, 32*(2), 263-271.

Covich, A. P., Fritz, S. C., Lamb, P. J., Marzolf, R. D., Matthews, W. J., Poiani, K. A., . . . Winter, T. C. (1997). Potential effects of climate change on aquatic ecosystems of the Great Plains of North America. *Hydrological Processes, 11*(8), 993-1021.

Driver, E. A., & Peden, D. G. (1977). The chemistry of surface water in prairie ponds. *Hydrobiologia, 53*(1), 33-48.

Evans, D. H. (2008). Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. [Journal Article]. *295*(2), R704-R713. doi: 10.1152/ajpregu.90337.2008

Ferrari, M. C. O., Messier, F., & Chivers, D. P. (2006). The nose knows: minnows determine predator proximity and density through detection of predator odours. *Animal Behaviour, 72*(4), 927-932.

Ferrari, M. C. O., Trowell, J. J., Brown, G. E., & Chivers, D. P. (2005). The role of learning in the development of threat-sensitive predator avoidance by fathead minnows. *Animal Behaviour, 70*(4), 777-784.

Ferrari, M. C. O., Wisenden, B. D., & Chivers, D. P. (2010). Chemical ecology of predator-prey interactions in aquatic ecosystems: A review and prospectus. *Canadian Journal of Zoology, 88*(7), 698-724.

Flower, R. J. (2001). Change, stress, sustainability and aquatic ecosystem resilience in North African wetland lakes during the 20th century: An introduction to integrated biodiversity studies within the CASSARINA Project. *Aquatic Ecology, 35*(3-4), 261-280.

Fu, S. J., Zeng, L. Q., Li, X. M., Pang, X., Cao, Z. D., Peng, J. L., & Wang, Y. X. (2009). The behavioural, digestive and metabolic characteristics of fishes with different foraging strategies. *Journal of Experimental Biology, 212*(14), 2296-2302.

Hammer, U. T. (1978a). The saline lakes of Saskatchewan I. Background and rationale for saline lakes research. *Internationale Revue der gesamten Hydrobiologie und Hydrographie, 63*(Journal Article), 173-177.

Hammer, U. T. (1978b). The saline lakes of Saskatchewan III. Chemical characterization. *Internationale Revue der gesamten Hydrobiologie und Hydrographie, 63*(Journal Article), 311-335.

Hammer, U. T. (1993). Zooplankton distribution and abundance in saline lakes of Alberta and Saskatchewan, Canada. [Article]. *International Journal of Salt Lake Research, 2*(2), 111-132. doi: 10.1007/bf02905904

Hammer, U. T., Sheard, J. S., & Kranabetter, J. (1990). Distribution and abundance of littoral benthic fauna in Canadian prairie saline lakes. *Hydrobiologia, 197*(1), 173-192.

Helfman, G. S. (1989). Threat-sensitive predator avoidance in damselfish-trumpetfish interactions. *Behavioral Ecology and Sociobiology, 24*(1), 47-58.

Hinga, K. R. (2002). Effects of pH on coastal marine phytoplankton. *Marine Ecology Progress Series, 238*, 281-300.

Hoover, Z., Ferrari, M. C. O., & Chivers, D. P. (2013). The effects of sub-lethal salinity concentrations on the anti-predator responses of fathead minnows. *Chemosphere, 90*(3), 1047-1052.

Hoover, Z., Weisgerber, J. N., Pollock, M. S., Chivers, D. P., & Ferrari, M. C. O. (2013). Sub-lethal increases in salinity affect reproduction in fathead minnows. *Science of the Total Environment, 463–464*, 334-339. doi: http://dx.doi.org/10.1016/j.scitotenv.2013.06.046

Hutchinson, T. H., Yokota, H., Hagino, S., & Ozato, K. (2003). Development of fish tests for endocrine disruptors. *Pure and Applied Chemistry, 75*(11-12), 2343-2353.

Ingersoll, C. G., Dwyer, F. J., Burch, S. A., Nelson, M. K., Buckler, D. R., & Hunn, J. B. (1992). The use of freshwater and saltwater animals to distinguish between the toxic effects of salinity and contaminants in irrigation drain water. *Environmental Toxicology and Chemistry, 11*(4), 503-511.

IPCC. (2014). Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. In Core Writing Team, R. K. Pachauri & L. A. Meyer (Eds.), (pp. 151). Geneva, Switzerland: IPCC.

Jacobsen, L., Skov, C., Koed, A., & Berg, S. (2007). Short-term salinity tolerance of northern pike, Esox lucius, fry, related to temperature and size. *Fisheries Management and Ecology, 14*(5), 303-308.

James, K. R., Cant, B., & Ryan, T. (2003). Responses of freshwater biota to rising salinity levels and implications for saline water management: A review. *Australian Journal of Botany, 51*(6), 703-713.

Kaushal, S. S., Groffman, P. M., Likens, G. E., Belt, K. T., Stack, W. P., Kelly, V. R., . . . Fisher, G. T. (2005). Increased salinization of fresh water in the Northeastern United States. *Proceedings of the National Academy of Sciences of the United States of America, 102*(38), 13517-13520.

Kefford, B. J., Papas, P. J., Metzeling, L., & Nugegoda, D. (2004). Do laboratory salinity tolerances of freshwater animals correspond with their field salinity? *Environmental Pollution, 129*(3), 355-362.

Kusch, R. C., Mirza, R. S., & Chivers, D. P. (2004). Making sense of predator scents: Investigating the sophistication of predator assessment abilities of fathead minnows. *Behavioral Ecology and Sociobiology, 55*(6), 551-555.

Lake, P. S. (2011). *Drought and aquatic ecosystems: effects and responses*. Chichester, West Sussex; Hoboken, N.J.: Wiley- Blackwell.

Landis, W. G., & Yu, M. (2004). *Introduction to environmental toxicology: Impacts of chemicals upon ecological systems* (3rd ed.). Boca Raton, FL; London: Lewis Publishers.

Last, W. M. (1992). Chemical composition of saline and subsaline lakes of the northern Great Plains, western Canada. *International Journal of Salt Lake Research, 1*(2), 47-76.

Last, W. M., & Slezak, L. A. (1988). The salt lakes of western Canada: A paleolimnological overview. *Hydrobiologia, 158*(1), 301-316.

Lavado, R., Maryoung, L. A., & Schlenk, D. (2011). Hypersalinity acclimation increases the toxicity of the insecticide phorate in coho salmon (Oncorhynchus kisutch). *Environmental Science and Technology, 45*(10), 4623-4629.

Leduc, A. O. H. C., Ferrari, M. C. O., Kelly, J. M., & Brown, G. E. (2004). Learning to recognize novel predators under weakly acidic conditions: The effects of reduced pH on acquired predator recognition by juvenile rainbow trout. *Chemoecology, 14*(2), 107-112.

Leduc, A. O. H. C., Kelly, J. M., & Brown, G. E. (2004). Detection of conspecific alarm cues by juvenile salmonids under neutral and weakly acidic conditions: Laboratory and field tests. *Oecologia, 139*(2), 318-324.

Leduc, A. O. H. C., Roh, E., Macnaughton, C. J., Benz, F., Rosenfeld, J., & Brown, G. E. (2010). Ambient pH and the response to chemical alarm cues in juvenile Atlantic salmon: Mechanisms of reduced behavioral responses. *Transactions of the American Fisheries Society, 139*(1), 117-128.

Lima, S. L., & Dill, L. M. (1990). Behavioral decisions made under the risk of predation: a review and prospectus. *Canadian Journal of Zoology, 68*(4), 619-640.

Luz, R. K., Martínez-Álvarez, R. M., De Pedro, N., & Delgado, M. J. (2008). Growth, food intake regulation and metabolic adaptations in goldfish (Carassius auratus) exposed to different salinities. *Aquaculture, 276*(1-4), 171-178.

Martínez-Palacios, C. A., Salgado-García, R. L., Racotta, I. S., Campos-Mendoza, A., & Ross, L. G. (2008). Effects of salinity on eggs, larvae, and juveniles of blacknose silversides from Lake Chapala, Mexico. [Article]. *North American Journal of Aquaculture, 70*(1), 12-19. doi: 10.1577/a06-026.1

Martinovic-Weigelt, D., Ekman, D. R., Villeneuve, D. L., James, C. M., Teng, Q., Collette, T. W., & Ankley, G. T. (2012). Fishy aroma of social status: Urinary chemo-signalling of territoriality in male fathead minnows (Pimephales promelas). *PLoS ONE, 7*(11).

Mathis, A., & Smith, R. J. F. (1993). Chemical alarm signals increase the survival time of fathead minnows (Pimephales promelas) during encounters with northern pike (Esox Lucius). *Behavioral Ecology, 4*(3), 260-265.

McMillan, V. E., & Smith, R. J. F. (1974). Agonistic and reproductive behaviour of the fathead minnow (Pimephales promelas Rafinesque). *Zeitschrift für Tierpsychologie, 34*(1), 25-58. doi: 10.1111/j.1439-0310.1974.tb01788.x

Mirza, R. S., & Chivers, D. P. (2001). Do chemical alarm signals enhance survival of aquatic vertebrates? An Analysis of the Current Research Paradigm. In A. Marchlewska-Koj, J. J. Lepri & D. Müller-Schwarze (Eds.), *Chemical Signals in Vertebrates 9* (pp. 19-26). Boston, MA: Springer US.

Mojazi Amiri, B., Baker, D. W., Morgan, J. D., & Brauner, C. J. (2009). Size dependent early salinity tolerance in two sizes of juvenile white sturgeon, Acipenser transmontanus. *Aquaculture, 286*(1-2), 121-126.

Morel, F. M. M., & Hering, J. G. (1993). *Principles and applications of aquatic chemistry*. New York: New York : Wiley.

Mount, D. R., Gulley, D. D., Hockett, J. R., Garrison, T. D., & Evans, J. M. (1997). Statistical models to predict the toxicity of major ions to Ceriodaphnia dubia, Daphnia magna and Pimephales promelas (fathead minnows). *Environmental Toxicology and Chemistry, 16*(10), 2009-2019.

Noyes, P. D., McElwee, M. K., Miller, H. D., Clark, B. W., Van Tiem, L. A., Walcott, K. C., . . . Levin, E. D. (2009). The toxicology of climate change: Environmental contaminants in a warming world. *Environment International, 35*(6), 971-986.

OECD. (1992). OECD Guidelines for Testing of Chemicals: Fish acute toxicity test. Publication 203: Organisation for Economic Cooperation and Development.

OECD. (2009). OECD Guidelines for testing of chemicals: Fish short term reproduction assay. Publication 229: Organisation for Economic Cooperation and Development.

Piscart, C., Usseglio-Polatera, P., Moreteau, J. C., & Beisel, J. N. (2006). The role of salinity in the selection of biological traits of freshwater invertebrates. *Archiv fur Hydrobiologie, 166*(2), 185-198.

Pistole, D. H., Peles, J. D., & Taylor, K. (2008). Influence of metal concentrations, percent salinity, and length of exposure on the metabolic rate of fathead minnows (Pimephales promelas). *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, 148*(1), 48-52.

Pollock, M. S., Fisher, S. E., Squires, A. J., Pollock, R. J., Chivers, D. P., & Dube, M. G. (2008). Relative body size influences breeding propensity in fathead minnows: Implications for ecotoxicology testing procedure. *Water Quality Research Journal of Canada, 43*(4), 257-264.

Pollock, M. S., Pollock, R. J., & Chivers, D. P. (2006). Effects of body size, body condition, and breeding state on responses to alarm cues by fathead minnows. *Canadian Journal of Zoology, 84*(9), 1351-1357.

Pratchett, M. S., Bay, L. K., Gehrke, P. C., Koehn, J. D., Osborne, K., Pressey, R. L., . . . Wachenfeld, D. (2011). Contribution of climate change to degradation and loss of critical fish habitats in Australian marine and freshwater environments. *Marine and Freshwater Research, 62*(9), 1062-1081.

R Development Core Team. (2011). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

R Development Core Team. (2015). R: A language and environment for statistical computing (Version 3.2.3). Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

Rasband, W. S. (1997-2012). ImageJ. Bethesda, Maryland, USA: U.S. National Insitutes of Health. Retrieved from http://imagej.nih.gov/ij/

Rawson, D. S., & Moore, J. E. (1944). The Saline Lakes of Saskatchewan. *Canadian Journal of Research, 22*(Journal Article), 141-201.

Relyea, R. A., & Mills, N. (2001). Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (Hyla versicolor). *Proceedings of the National Academy of Sciences of the United States of America, 98*(5), 2491-2496.

Rickwood, C. J., Dubé, M. G., Hewitt, L. M., Kovacs, T. G., Parrott, J. L., & MacLatchy, D. L. (2006). Use of paired fathead minnow (Pimephales promelas) reproductive test. Part 1: Assessing biological effects of final bleached kraft pulp mill effluent using a mobile bioassay trailer system. *Environmental Toxicology and Chemistry, 25*(7), 1836-1846.

Rosengrave, P., Taylor, H., Montgomerie, R., Metcalf, V., McBride, K., & Gemmell, N. J. (2009). Chemical composition of seminal and ovarian fluids of chinook salmon (Oncorhynchus tshawytscha) and their effects on sperm motility traits. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 152*(1), 123-129.

Ross, S. (2001). *The inland fishes of Mississippi*. Mississippi: Mississippi Department of Wildlife, Fisheries, and Parks.

RStudio Team. (2015). RStudio: Integrated Development for R (Version 0.99.491). Boston, MA: RStudio Inc. Retrieved from http://www.rstudio.com/

Stacey, N., & Sorensen, P. (2005). Reproductive Pheromones. *Fish Physiology*, 24

Stekoll, M. S., Smoker, W. W., Failor-Rounds, B. J., Wang, I. A., & Joyce, V. J. (2009). Response of the early developmental stages of hatchery reared salmonids to major ions in a simulated mine effluent. *Aquaculture, 298*(1-2), 172-181.

Trombulak, S. C., & Frissell, C. A. (2000). Review of ecological effects of roads on terrestrial and aquatic communities. *Conservation Biology, 14*(1), 18-30.

USEPA. (1987). Guidelines for the culture of fathead minnows Pimephales promelas for use in toxicity tests. EPA/600/S3-87/001. Duluth, MN.

USEPA. (2002). Short-term test method for assessing the reproductive toxicity of endocrine-disrupting chemicals using the fathead minnow (Pimephales promelas). EPA 600/R-01/067. Duluth, MN.

Weber-Scannell, P. K., & Duffy, L. K. (2007). Effects of total dissolved solids on aquatic organisms: A review of literature and recommendation for salmonid species. *American Journal of Environmental Sciences, 3*(1), 1-6.

Weber, D. N., & Bannerman, R. (2004). Relationships between impervious surfaces within a watershed and measures of reproduction in fathead minnows (Pimephales promelas). *Hydrobiologia, 525*(1-3), 215-228.

Wedderburn, S. D., Barnes, T. C., & Hillyard, K. A. (2014). Shifts in fish assemblages indicate failed recovery of threatened species following prolonged drought in terminating lakes of the Murray–Darling Basin, Australia. [journal article]. *Hydrobiologia, 730*(1), 179-190. doi: 10.1007/s10750-014-1836-2

Whiterod, N. R., & Walker, K. F. (2006). Will rising salinity in the Murray-Darling basin affect common carp (Cyprinus carpio L.)? *Marine and Freshwater Research, 57*(8), 817-823.

Williams, W. D. (1964). A contribution to lake typology in Victoria, Australia. *Verhandlugen Internationale Vereinigung fur Limnologie, 15*(Journal Article), 158-163.

Williams, W. D. (1987). Salinization of rivers and streams: an important environmental hazard. *Ambio, 16*(4), 180-185.

Williams, W. D. (2001). Anthropogenic salinisation of inland waters. *Hydrobiologia, 466*(Journal Article), 329-337.

Zimmer, K. D., Hanson, M. A., & Butler, M. G. (2002). Effects of fathead minnows and restoration on prairie wetland ecosystems. *Freshwater Biology, 47*(11), 2071-2086.

Appendix A: Methods for Study 1

A.1 General

The majority of the study protocol for the fish component of the study will be drawn directly from the OECD guidelines for testing of chemicals on fishes (OECD, 1992). However, as done in several studies, portions of the protocol will be modified to better fit the questions asked.

A.2 Fish test species

Following discussion with Fisheries and Oceans Canada, the Saskatchewan Watershed authority, Ministry of the Environment, and the University of Saskatchewan it was decided that the test species to be used in the current study will be walleye, pike, and a yet to be determined forage species (*e.g.* minnow, shiner, etc.). Walleye are the primary species of concern with respect to Lake Lenore, and as such, is the species of greatest concern to Fisheries and Oceans. Though a concern over the laboratory survival of the walleye was raised, its importance in Lake Lenore makes an attempt with this species the preferred option. If the walleye appears to be an unsuitable species, the yellow perch was suggested as a surrogate. Given the fact that perch are closely related to walleye and appear to have similar salinity tolerance to walleye (Rawson & Moore, 1944), this seems a logical choice. Pike was also selected as a species important to Lake Lenore, and along with a yet to be determined forage species, will comprise the three species used in the current study.

Forage fish selection will be based on early spring sampling of the Lake Lenore fish community by Fisheries and Oceans and Ministry of the Environment. A species of forage fish will be selected that is abundant in the Lake and/or represents a majority of the species present (*i.e.* closely related or an ecological guild member).

A.2.1 Fish sourcing and housing

Given the size of the test tanks are approximately 10 l it was decided that the walleye and pike used would be young-of-the-year, approximately 10 g in size. Jennifer Merkowsky representing the

Ministry of the Environment will source the fish from provincial hatcheries and initiate coordination to get the fishes safely to the University of Saskatchewan Laboratory. Dependent on forage fish selection, stock may be obtained from naturally occurring waterbodies or laboratory supply companies.

Once delivered to the laboratory, fishes will be initially housed in living streams (Model # LS-700- 215 cm L x 60 cm W x 55 cm D, 680 operating liters). As per the OECD protocol, fishes will be held at a 16-hour photoperiod, in at least 80% oxygen saturation, and be fed brine shrimp (*Artemia* spp.) twice daily. Fishes will be allotted a 48-hour settling in period during which time health will be visually assessed and any dead fish removed. Following the settling in period, fish will be held in the same tank, in the same conditions for a week to ensure the populations are healthy and under minimal stress prior to the study outset. Mortalities greater than 10% during this period may lead to rejection of the entire brood unless a problem can be identified and rectified. Mortalities less than 10% are acceptable.

A.2.2 Fish exposure

Fishes will be placed in individual tanks (number of individuals per tank dependent on size of fish) containing an air stone and lid, and held under static conditions (i.e. no flow through or addition of water). Immediately prior to being placed in tanks, length, mass, and general health of fish will be recorded to ensure subject size is equal among treatments. Fish will be held in experimental tanks for a period of 24 hours (all tanks at control levels) to allow acclimation and reduce stress prior to exposure. During this period, and for the remainder of the study, fish will be deprived of food.

Subjects will be exposed to one of seven concentrations of synthetically derived TDS, a series including a control (250 ppm) followed by 1000, 2000, 4000, 6000, 8000, 16000 ppm. Given the fact that presence and ratio of specific ions is known to impact survivability is it recommended that the ion ratio used be derived from samples taken from Lake Lenore, thus adding to ecological relevance.

Similarly, given the importance of temperature with respect to TDS toxicity (Jacobsen et al., 2007) a temperature will be used that is relevant to the timing of exposure in Lake Lenore. Historic temperature data will be used to determine the average temperature found in Lake Lenore in early spring (the timing of flow from Lake Houghton to Lake Lenore), thus adding ecological relevance to the study outcome.

The OECD protocol recommends immediate exposure following the 24-hour acclimation period (*i.e.* adding a concentrated sample into each tank with no acclimation period). However, due to the fact that acclimation period is known to impact survivability (Martínez-Palacios et al., 2008), it is recommended that the ion concentration be slowly added over a 48-hour period until full concentration is reached.

Sample size used will be dependent on the availability of fish, but a minimum sample size of ten tanks at each concentration is recommended. The OECD protocol recommends a sample size between 3 and 5. However, with a greater sample size, statistical power will be increased with minimal increased effort.

The standard exposure period consists of 96 hours, during which time fish are checked and mortalities recorded every 12 hours. Daily water quality checks will be conducted on 20% of the tanks within each treatment group. Water quality will include temperature, dissolved oxygen, pH, ammonia, and conductivity.

A.2.3 Fish behaviour

Along with mortality, each fish will be observed for five minutes daily to determine if behaviour is being impacted by the change in TDS. To create consistent observational data, an observer will sit two meters from the tank and record observations at the same time daily. Each day the order in which tanks are observed will be determined arbitrarily via a random numbers table. The most common behavioural measure used in such studies is time spent moving and position in tank (Luz et al., 2008; Whiterod & Walker, 2006). Thus, tanks will be divided into three equal vertical sections with the location of the fish within the tank being recorded every 10 seconds. A stop watch will also be used to calculate the time spent moving within the observation period. Both of these measures are common variables used to assess stress in fishes (Luz et al., 2008; Whiterod & Walker, 2006).

A.2.4 Exposure end

On the final day of exposure fish will be removed from the tanks and euthanized using an overdose of MS-222 (animal care protocol pending), followed by recording of weight, length, and

general health. At this time, it is also recommended that samples of tissue from liver, kidneys, muscles, and gonads be collected and frozen for future analysis.

Appendix B: Salinity Preparation for Study 1

The following masses (cumulative) of salts were added over a 24 h period to reach the following nominal concentrations used in Study 1:

		250ppm	1000ppm	2000ppm	4000ppm	6000ppm	8000ppm	16000ppm
Sodium Carbonate	Na2CO3	0.0451	0.1805	0.3611	0.7222	1.0833	1.4444	2.8887
Potassium Chloride	KCl	0.1038	0.4153	0.8306	1.6613	2.4919	3.3226	6.6452
Sodium Bicarbonate	NaHCO3	0.3153	1.2614	2.5227	5.0455	7.5682	10.0909	20.1819
Magnesium Sulfate	MgSO4	1.3198	5.2792	10.5584	21.1169	31.6753	42.2338	84.4675
Calcium Sulfate Dihydrate	CaSO4*2H2O	0.1279	0.5117	1.0233	2.0466	3.0699	4.0932	8.1864
Calcium Chloride Dihydrate	CaCl2*2H2O	0.0185	0.0740	0.1481	0.2961	0.4442	0.5922	1.1844
Sodium Sulfate	Na2SO4	0.3508	1.4031	2.8063	5.6125	8.4188	11.2251	22.4502